# CITRUS TRISTEZA VIRUS: MOLECULAR CHARACTERIZATION OF ISOLATES FOR USE IN MILD STRAIN CROSS PROTECTION, LOCALIZATION OF THE 5'-TERMINUS AND HETEROLOGOUS ENCAPSIDATION.

By

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To my best friend and beloved wife Olimpia Emilia, and our joyfullness: Luisana and Francisco.

To my parents, Francisco and Silfa, for their values and love, and for teaching me that life goals are not dreams and can be made true.

With love to my sister and brother.

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By

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Chairman: Richard F. Lee.
Co-chairman: Charles L. Niblett
Major Department: Plant Pathology.

The purpose of this research was to validate the use of recently developed methods which permit selective differentiation of mild and severe strains of citrus tristeza virus (CTV) to speed the selection of mild CTV strains potentially useful for mild strain cross protection (MSCP) against citrus tristeza virus. The molecular evaluation for differentiation of CTV strains can be applied for the detection of mild and severe strains from the original *in planta* culture collected from surviving trees in CTV infected areas as well as from single aphid transmitted sub-isolates. The application of these methods of CTV strain differentiation should enable faster and better selection of mild strains which may be useful for MSCP programs.

The localization of the capsid protein (CP) and minor CP in relation to the genomic RNA termini of purified CTV virions *in planta* was examined, regarding the polar architecture of CTV virions, by specifically immunocapturing one termini with polyclonal antisera prepared against the bacterial expressed CTV minor CP, p27. Immuno-capture reverse transcriptase-PCR (IC-RT-PCR) conditions were optimized for the amplification of the first 510 nt at the 5'- terminus, and the last 899 nt at the 3'-terminus of the CTV genome. The 5'-terminus of CTV was consistently amplified when virions were immunocaptured using the p27 CP specific antiserum. CTV was purified, sonicated to fragment the virions, and then fractionated on a 10-40 % rate zonal sucrose gradient. Gradient fractions were tested by ELISA and used to recover the fragments which were then tested for termini amplification by IC-RT-PCR. The results demonstrate that the minor CP, p27, encapsidates the 5'- terminus of the CTV genomic RNA.

Heterologous encapsidation studies were performed by inoculating two mild isolates of CTV, T30a and T11a, into transgenic citrus plants which were expressing the CP gene of a T36 decline isolate of CTV. Heterologous encapsidation of the genome of the mild isolates by the CP of the severe strain, T36, was examined by using the MCA13 antisera specific to severe Florida isolates, and by selectively immunocapturing virions by the p27 CP using the p27 specific antiserum, UF 34, then IC-RT-PCR followed by strain specific hybridization probes. Two of twenty six plants were MCA13 positive, showing heterologous encapsidation between virions and the T36 CP expressed *in planta.*

# *CHAPTER 1*
# CITRUS TRISTEZA DISEASE: THE CAUSAL AGENT AND VECTOR

## Citrus tristeza disease

Tristeza disease is caused by Citrus tristeza virus (CTV) a member of the genus Closterovirus within the family *Closteroviridae* (Mayo and Pringle 1997; van Regermortel *et al.* 2000). Tristeza is one of the most devastating citrus diseases in tropical and semi-tropical regions worldwide where citrus is grown. “Tristeza”, meaning sadness in Spanish and Portuguese, was the name was given to the disease in South America to describe the collapse of citrus scions when grafted on sour orange (*Citrus aurantium* L.) rootstock (Bar-Joseph *et al.* 1989). The disease was first described by Bitancourt (1940, 1941), and the viral nature of the decline on sour orange was realized after transmission of the syndrome by the aphid, *Toxoptera citricida*, (Meneghini 1946) and tissue grafting (Wallace and Fawcett 1947). The host range of CTV is limited to *Citrus* and related genera and to some species in genus *Passiflora* (Müller *et al.* 1974). Recently, *Nicotiana benthamiana* protoplasts have been experimentally infected with CTV (Navas-Castillo *et al.* 1997).

CTV expresses a multitude of symptoms on various hosts. A standardized host range of five indicator plants has been established to determine the biological characteristics of CTV isolates (Garnsey *et al.* 1987). Mexican lime (*Citrus aurantifolia* (Christm.) Swing.) is used as a CTV-sensitive universal indicator; sour orange seedlings are used for detection of seedling yellows; sweet orange (*Citrus sinensis* (L.) Osb.)

grafted on sour orange rootstock plants are used for the detection of decline-inducing strains; Duncan grapefruit seedlings are used for the detection of stem pitting on grapefruit (*Citrus paradisi* Macf.), and Madam Vinous sweet orange seedlings are used for the detection of stem pitting on sweet orange.

In general, the most debilitating diseases caused by CTV are of two general types: 1) decline or death of scions on sour orange, and 2) stem pitting on scions regardless of the rootstock (Lee, *et al.* 1994). The first disease, decline on sour orange rootstock, is often expressed as a rapid death of sweet orange, grapefruit, or mandarin scions when grafted on sour orange rootstock (Fig. 1-1.A) while in other cases, the decline and death occur over a period of several months. Trees undergoing decline on sour orange rootstock due to CTV often have a bulge immediately above the bud union and characteristic "honeycombing" occurring on the phloem-side of a bark patch cut through the bud union over the sour orange rootstock (Fig. 1-1.B and 1-1.C). The second disease, stem pitting, is considered the most damaging. Stem pitting reduces vigor, yield and fruit quality of sweet orange and/or grapefruit and/or lime regardless of the rootstock (Fig. 1-1.D, 1-1.E), and the damage cannot be avoided by using CTV-tolerant rootstocks. Other symptoms often associated with stem pitting phenotypes are vein clearing, vein corking (Fig. 1-1.F, 1-1.G), and leaf cupping. Mild phenotypes of CTV induce minor vein clearing and some stem pitting in Mexican lime, but do not express symptoms in commercial citrus varieties. About 300 isolates representing the known differences symptom expression and serological properties have been collected from around the world and stored *in planta* at the Collection of Exotic Citrus Diseases in Beltsville, MD,

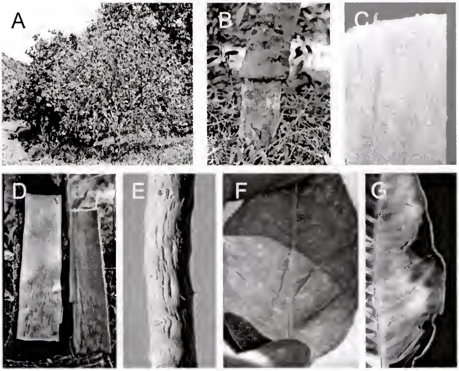


Fig. 1-1. Citrus tristeza virus symptoms. A) Decline of Valencia sweet orange grafted on sour orange rootstock. B) Bulge above the bud union of sweet orange grafted on sour orange rootstock. C) Phloem side of a sour orange bark piece cut near the bud union showing "honeycombing". There are corresponding bristles or pegs protruding from the xylem tissue beneath the bark piece (magnification 2X). D) Stem pitting on Volkamer lemon (*Citrus volkameriana* Ten. and Pasq.) rootstock of a four year old Valencia tree. E) Stem pitting on a Valencia sweet orange branch due to CTV isolate B-249. F) Vein clearing on Mexican lime leaf due to infection by CTV, isolate B248. G) Vein corking on Valencia sweet orange leave due to CTV isolate B-249. All pictures are by the author and from Venezuela.

USA (Garnsey *et al.* 1987,1991). Most of those isolates have been characterized using the standardized host range (Garnsey *et al.*, 1991), and they are available for research use.

Early histological and cytological studies on tissue and cells infected by CTV showed that citrus combinations on sour orange rootstock accumulated abnormally high levels of carbohydrates above the budunion (Schneider 1957a, 1957b, 1959). Furthermore, hyperplasia and dead cells in the phloem were observed at the bud union, suggesting a hypersensitivity reaction of the scion with the sour orange rootstock upon infection with CTV (Schneider 1957b, 1959). To date, the nature of this reaction is uncharacterized and has not been studied at the physiological and molecular level. More recent histological studies confirmed Schneider's observations and indicated an association among the biological activity of several CTV strains, the number of inclusion body formed, and the amount of histological damage in the phloem (Brlansky 1990; Ochoa *et al.* 1994; Lin *et al.* 2000).

CTV exists as mixtures of strains, or populations, in most field isolates because of the perennial nature and vegetative propagation of citrus which perpetuates existing CTV infections. Additionally, trees in the field are exposed to multiple infections by aphids. Divergent CTV genotypes can be spread over long distances by movement of infected propagation material, thus providing a means for new CTV genotypes to spread into new areas. Several isolates of CTV with significant biological differences in symptoms and serological properties have been well characterized (Bar-Joseph *et al.* 1984; Bar-Joseph and Lee 1989 Rocha-Peña *et al.* 1991, 1992; Karasev *et al.* 1995; Vives *et al.* 1999; Albiach *et al.* 2000). Segregation of the CTV strains from field isolates has been shown by analysis of dsRNA (Moreno *et al.* 1991), hybridization with sequence specific probes

(Cevik 1995 ; Cevik *et al.* 1996), single–strand conformation polymorphism (SSCP) (Febres *et al.* 1995; Rubio *et al.* 1996), PCR colony hybridization (Genc, 1998), and single aphid transmissions (Broadbent *et al.* 1996; Marais *et al.* 2000; Tsai, Liu *et al* 2000).

## The CTV genome

CTV has the largest RNA genome of all the viruses infecting plants (Bar-Joseph *et al.* 1984). The genome consists of a single-stranded positive-sense RNA of 19,296 nt for the Florida T36 isolate (Karasev *et al.* 1995), and 19,259 nt for both T385 (Spain) and T30 (Florida) isolates (Albiach-Martí *et al.* 2000). The CTV virions consist of flexuous particles about 2,000 x 11 nm in size with the capsid protein (CP) encapsidating 95 percent of the RNA genome and the homolog minor CP, p27, encapsidating about 5 per cent of one end of genome. The p27 minor CP encapsidates 75-85 nm of one termini, and the p25 CP encapsidates about 1,900 nm of the CTV virion (Febres *et al.* 1996).

The CTV genome is organized into 12 open reading frames (ORFs) potentially encoding up to 19 protein products (Pappu *et al.* 1994a; Karasev *et al.* 1995;). Like other viruses within the *Closteroviridae*, two characteristic gene blocks are distinguishable at the 5'- and 3'- regions (Fig. 1-2). The 5' terminus contains the first gene block which contains a polyprotein module that includes two proteases domains, methyltransferase, helicase-like domain, an RNA-dependent RNA polymerase expressed by a +1 frameshift to p18 like a (RdRp) domain, and the p33 gene having an unknown function (Karasev *et al.* 1995; Agranovsky 1995). Infectivity tests using an infectious clone indicated that

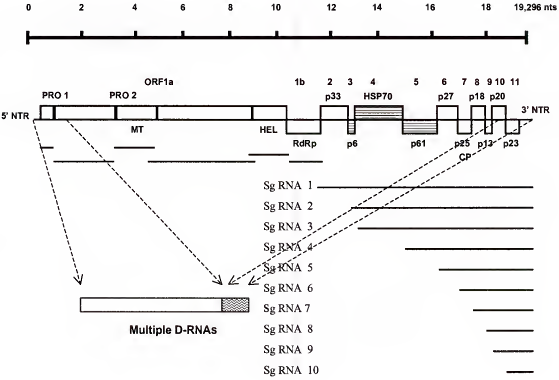


Fig. 1-2. Schematic representation of the gene expression and genome organization of citrus tristeza virus. The ORFs 1 to 11 are represented by boxes and the putative domains on ORF 1a, 1b are indicated: papain-like proteases 1 and 2 (PRO), methyltransferase (MT), Helicase (HEL), and RNA-dependent RNA polymerase (RdRp). The two 3'-modules are differentially highlighted as well the two CPs. The genomic and subgenomic RNAs are shown by solid lines at the bottom-right. The size, in kilobases, is indicated by the scale at the top. The defective-RNA recombination strategy is show by dashed lines at, the bottom left.

CTV clones in which the p33 was deleted can still infect protoplasts (Satyanarayana *et al.*, 2000). Computer analysis of the overlaping region of ORF 1a and ORF 1b from CTV reveals a highly conserved region with 84-100 percent nucleotide sequence identity among the isolates compared, suggesting the possible role of the stem loop structure for pausing the ribosome during frameshifting (Cevik *et al.* 1999).

The second gene block of CTV occurs at the CTV 3'-end and encodes 10 ORFs expressed through 3'-coterminal subgenomic mRNAs (sgRNAs) (Hilf *et al.* 1995; Karasev *et al.* 1997) (Fig. 1-2). The 5'-termini of some sgRNA were recently studied (Ayllón *et al.* 2000). The sgRNAs of ORFs 7 (p25), 8 (p18), and 9 (p13) were found to have leader sequences of 37, 38 and 10 nt , respectively.

The CTV 3'-end gene block can be grouped into two modules. The upstream 3'-module is the movement-assembly module that encodes the 6 KDa, HSP70 homolog (p65), p61, p27 and p25. The 6 KDa protein has been proposed to function as a membrane anchor because of its hydrophobicity and proximity to the p65 (Dolja *et al.* 1994). The HSP70 homolog, p65, and p61 proteins are required for the efficient assembly of virions in addition to the two CPs (Satyanarayana *et al.* 2000). Deletions in the p65 and the p61 genes produced a minimum of viable virions. Similarly, deletion of either the p27 (minor CP) or p25 CP genes resulted in the assembly of short particles (Satyanarayana *et al.* 2000). The 3'-terminal module is composed of the p18, p13, p20 and p23 genes. Recently a study of the homologous and heterologous interactions of p27, p25, p23 and p20 using the yeast two–hybrid system assay disclosed an affinity of the p20 for itself (Gowda *et al.* 2000). This study indicated a clear association of p20 with the amorphous inclusion bodies which are characteristic of CTV infections. The p23 has been shown to have an affinity for single-stranded RNA as studied by gel retardation, UV crosslinking and competition assays (Lopez *et al.* 2000). Although the precise function of the RNA-binding by p23 is still unknown, it seems likely that an early accumulation of this protein serves a regulatory function. Infectious mutant clones having the p18 and p13 genes deleted still accumulated RNA after being serially passaged in *N. benthamiana* protoplasts (Satyanarayana *et al.* 2000).

Comprehensive computer analysis of CTV genomic sequences revealed a unique nucleotide identity along the CTV genome. A difference of 42.5 percent in nucleotide identity within the 5'- end of T36 in respect to T385, VT and SY586 has been reported, whereas the differences progressively decreased toward the 3'- terminus (Mawassi *et al.* 1996; Bar-Joseph *et al.* 1997; Lopez *et al.* 1998; Vives *et al.* 1999). The remarkable variability observed between the 5' end and the 3'end, might be explained by a parallel pathway of evolution between the two termini, followed by further modular recombination among blocks of the CTV genome which generated a virus that looks like a natural recombinant. (Dolja *et al.* 1994; Bar-Joseph *et al.* 1997).

The presence of untranslated regions (UTR) of 107 and 275 nt at the 5'- and 3'-termini of the T36 strain, respectively, was first reported based on sequencing studies (Karasev et al. 1995). Over 97 percent identity was reported among the 3'- UTRs of the T385, T36, and SY586 strains, whereas the 5'-UTR of T385 had more variability with 67, 66.3, and 42.5 percent identity reported for VT, SY586 and T36 strains, respectively (Vives *et al.* 1999). Furthermore, a 96.3 percent identity between the 5'-UTR of T385 and T30 was reported, whereas a 99.6 percent identity was reported for the 3'-UTR

(Albiach-Martí *et al.* 2000). This is a strikingly high level of conservation of the 5'- and 3'- UTRs for two mild strains, which are geographically distant in their origin.

Defective RNAs (D-RNAs) are present as multiple species in CTV-infected plants. The D-RNAs consist of 5'- and 3'- termini of the genomic RNA with an internal deletion of the genomic RNA (Fig. 1-2) (Mawassi *et al.* 1995a, 1995b, 1995c, 1997). Plants infected with CTV often contain and accumulate a few D-RNAs at high concentrations, but it has been suggested that D-RNAs are not randomly produced. Hybridization analyses revealed that D-RNAs occur abundantly in plants infected with most CTV isolates. A process of selection for fitness of the D-RNA has been proposed based on minimal length conformation of 5'- and 3'- termini, spacing of the terminal sequences, and considering if continuous translation of the 5'- terminal occurs, apparently without its protein product (Mawassi *et al.* 2000). D-RNAs provide possible evidence for the natural-modular recombination of CTV.

Advances in our knowledge of the CTV genome organization and the availability of sequence information for several CTV strains now allows the application of the concepts of a virus species, phenotype and quasispecies to provide a better understanding of the complexity of CTV populations. A virus species is 'a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche'. (van Regermortel 1990). Phenotype refers to symptoms produced in a range of hosts (Albiach-Martí 2000). The term quasispecies for viruses refers to very related, but not identical viral genomes that replicate and recombine with high error rates and compete for fitness and selection (Domingo and Holland 1997; Roosinck 1997)

## The vector

The brown citrus aphid (BrCA), *Toxoptera citricida* Kirk., was first described by Kirkaldy (1907) and named *Aphis citricidus* (1935-1960), then called *Toxoptera citricidus* before being renamed to its current scientific name (Stoetzel 1994; Michaud 1998). A recent and complete description of this vector was reviewed by Halbert and Brown (1996). The aphid is thought to have originated in Southeast Asia, the same place that CTV originated (Kirkaldy 1907; Yokomi et al. 1994; Rocha-Peña *et al.* 1995a). A list of host plants from which the BrCA has been reported and also the natural enemies and entomopathogens has been compiled by Michaud (1998).

BrCA feeding is restricted mostly to tender citrus flushes. The BrCA population varies region by region as citrus growth is strongly influenced by rainfall, irrigation practices, pruning, and fertilization management. The life cycle and population dynamics of BCA were studied in Venezuela (Geraud 1979) following the introduction of the aphid in 1976 (Geraud 1976).

The BrCA transmits CTV in a semi-persistent manner (Bar-Joseph *et al.* 1979a). CTV is acquired and transmitted by BrCA with feeding times ranging from several minutes to several hours. The molecular mechanism by which the CTV is acquired and further transmitted is unknown.

## CTV in the Caribbean and cross protection

CTV is an important crop in Latin America and the Caribbean Basin. The events that occur before, during and after the CTV epidemics have been reviewed (Ochoa, F. *et al.* 1994; Rocha-Peña *et al.* 1998). The development of management strategies has helped keep the citrus groves productive despite the aggressiveness of the disease and its vector.

Certainly, the citrus rootstock management strategies and nucellar selection with use of mild strain cross protection have been proven to be effective under certain biological scenarios as in Brazil (Costa and Müller 1980). However, the virus and mainly the stem pitting variants, are an important constituent element of the CTV epidemics and should not be underestimated (Rocha-Peña *et al.* 1995a).

Mild strain cross protection (MSCP) has been defined as a phenomenon by which a mild strain of a virus is inoculated into a plant and prevents the expression of the symptoms of a severe strain of the same virus upon subsequent infection (Lee *et al.* 1987). The use of a microorganism *in planta* to protect against severe symptoms of another, place cross protection among the biological control strategies (Charudathan 1984). It was observed early that when a less virulent strain of a virus was inoculated before a second virulent strain of the same virus was introduced, the plant often did not express the symptoms of the severe challenging strain (Salaman 1933 and Kunkel 1934). The molecular mechanism or the virus-host molecular determinants which governs cross protection are unknown at present, but cross protection has been empirically developed as a biological control approach for management of viruses in tropical and subtropical crops (Gonsalves and Garnsey, 1989). Different mechanisms have been proposed to explain cross-protection (Abel, *et al.* 1986). In general, it has been hypothesized that the CP produced from the first viral infection then encapsidates the RNA of the challenging strain, minimizing its replication (de Zoeten and Fulton 1975). However, experiments performed with TMV mutants defective for CP questioned that hypothesis (Zaitlin 1976).

There are several factors to consider before using cross protection. The advantage, disadvantage, risks, limitations and characteristics of the virus, host and vector have been widely discussed by Fulton (1986) and Lee *et al.* (1987). There is a general consensus about the overall characteristics of a mild strain: there should be no synergistic activity with other viruses; the vectors should have a limited host range; the isolate must be stable and not prone to mutation; and commercial yields realized in spite of the presence of the severe strain (Fulton, 1986, Lee, *et al.*, 1987, and Van Vuuren, 1991).

The application of molecular techniques has provided valuable information about the CTV transcriptional strategy and genome organization. The existence of specific genetic sequences within *Closteroviridae* has aided the discovery process and allowed a molecular dissection of the CTV genes and their functions. Still the molecular host-pathogen and vector-pathogen interactions have not been discovered, and it is not yet possible to assign specific symptoms on citrus cultivars as due to particular viral sequences, nor has a sequence yet been found which influences transmissibility by *T. citricida.* There is now a general acceptance about the existence of a cellular RNA-surveillance mechanism which discriminates and removes non-host RNA (Dawson 1996; English *et al.* 1996; Gura 2000; Marx 2000). This should drive future research to explore the use of gene silencing for virus resistance, and possibly provide information about the CTV cross protection mechanism.

The purpose of this research was to validate the use of methods which permit selective differentiation of mild and severe strains of CTV to speed the selection of mild strains which may be potentially useful for mild strain cross protection (Chapter 2). Also the research was directed to trying to determine the nature of virion encapsidation with the p27 (Chapter 3), and to study the phenotypic mixing relationship between CTV virions and CP transgenes expressed *in planta* (Chapter 4).

## *CHAPTER 2*

# MOLECULAR CHARACTERIZATION OF FLORIDA CITRUS TRISTEZA VIRUS ISOLATES TO AID IN THE SELECTION OF ISOLATES WITH POTENTIAL USE IN MILD STRAIN CROSS PROTECTION

## Introduction

There are an estimated 15 million citrus trees on sour orange rootstock in Florida (Witzig and Pugh 1996) which are highly susceptible to decline-inducing strains of citrus tristeza virus (CTV-D). Previous work has demonstrated that some mild strains of CTV can minimize the symptoms induced by stem pitting strains (CTV-SP) when the mild strain is inoculated into the plant before the severe strain, a phenomenon often referred to as mild strain cross protection (MSCP) (Costa and Müller 1980; Lee *et al.* 1987; Gonsalves and Garnsey 1989; Powell, *et al.* 1992; Ochoa *et al* 1993; Fuchs *et al.* 1997). MSCP using selected mild strains has been shown to be useful to prolong the life expectancy of trees on sour orange rootstock in Florida (Yokomi *et al.* 1990; Rocha-Peña, 1990; Powell *et al.* 1999). The development of a better understanding of the basis of MSCP is an important long-term goal for the continued protection of citrus from CTV in Florida. However, the selection of mild strains useful for MSCP has been an empirical and time-consuming process. While there are many mild CTV strains, only a few have any cross protection ability, about one out of 200-300 (Lee *et al.* 1987 Lee *et al.* 1995; R. Lee and G. Müller, personal communication). In Florida, CTV mild strains for MSCP have been collected, mostly from surviving trees on sour orange rootstock from areas where CTV losses have been severe (Powell *et al.* 1992; Lee *et al.* 1995). Five mild CTV strains have been extensively tested for MSCP under field conditions in Florida (Powell *et al.* 1992; Rocha–Peña *et al.* 1992; Lee *et al.* 1995; Powell *et al.* 1999).

A better understanding of the CTV genome has resulted in progress being made on the development of rapid methods for CTV strain differentiation. This progress has been reviewed recently (Lee *et al.* 1996). A monoclonal antibody, MCA13, has been developed. It reacts selectively with most severe CTV strains, including decline (CTV-D) and stem pitting (CTV-SP) strains (Permar *et al.* 1990; Pappu *et al.* 1994b). MCA13 has been useful in Florida and the Caribbean Basin to determine if a CTV isolate contains a severe strain (Rocha–Peña *et al.* 1995a). However, isolates have been found which contain CTV-SP, yet the isolate does not react with MCA13 (Batista *et al.* 1996). Biological indexing (Lee *et al.* 1995) thus provides the ultimate determination of the biological activity of a CTV isolate.

Recently methods for strain differentiation of CTV have been developed based on nucleic acid sequence differences in the coat protein gene. The strain specific probe (SSP) method (Cevik 1995; Cevik *et al.* 1996) allows the differentiation of strains and enables detection of mixed infections containing severe CTV strains which may not be detected by serological testing or by biological indexing. The SSP methodology is based upon the micro-heterogeneity among coat protein gene of phenotypically diverse CTV strains and uses non-radioactive oligonucleotide probes (Cevik 1995; Cevik *et al.* 1996). Another method which has been useful to differentiate CTV strains is single-strand conformation polymorphism (SSCP) of the PCR products of the capsid protein (CP) or p27, the minor CP gene (Febres *et al.* 1995; Rubio *et al.* 1996).

The purpose of this study was to evaluate SSP and SSCP for CTV strain differentiation and to detect the presence of severe CTV strains in the isolates collected from surviving trees for evaluation for MSCP, and in single aphid transmitted sub-isolates from the original sources using *Toxoptera citricida.*

## Materials and methods

**CTV isolates.** The isolates characterized in this study were collected from surviving trees on sour orange rootstock from areas where CTV had caused severe decline in commercial groves in Florida. The isolates were maintained as *in planta* cultures in Madame Vinous sweet orange at the University of Florida's Citrus Research and Education Center, Lake Alfred. The selection and collection procedure has been described recently (Lee and Niblett 1999) and is briefly summarized here. The field isolates were established *in planta* on several plants, and some of these plants were graft challenged with several very severe Florida CTV strains, with the challenge buds remaining in the plant. If the challenged plant continues to grow for 2-3 flushes, the respective *in planta* culture for that isolate which has not been challenged was selected for evaluation for MSCP. The selected *in planta* isolates also were used as the source for single aphid transmissions (SAT) using *T. citricida,* being subjected to additional rounds of SAT with a 24-hour acquisition feeding and a 24-hour inoculation feed. These SAT sub-isolates were designated with a one hundred number followed by an extension number. The CTV strains T36 (CTV-QD) and T30 (CTV-mild) were used as controls for reference.

**DAS-Indirect ELISA.** Fresh, tender bark tissue samples were pulverized in liquid nitrogen and stored at -80° C until needed. Double antibody sandwich (DAS)-

indirect ELISA assays were performed as previously described (Bar-Joseph *et al.* 1979b; Garnsey and Cambra 1991). CREC 1053 (Rocha–Peña *et al.* 1992) from the CREC antiserum collection was used for coating. Goat polyclonal antibody G604 (R. Lee and K. Manjunath, unpublished) and the monoclonal antibody MCA13 (Permar *et al.* 1990) were used as secondary antibodies for broad spectrum and selective detection of severe CTV strains, respectively. Anti-goat and anti-mouse antibody-alkaline phosphatase conjugates (Sigma, St Louis, MO.) were used as appropriate. $OD_{405nm}$ values three times that of the healthy controls were considered to be positive for CTV. Some ELISA plates were stored at -20° C for later use with immunocapture reverse-transcriptase-PCR (IC-RT-PCR) (Nolasco, *et al.* 1993).

**Extraction of nucleic acids, IC-RT-PCR.** RNA extracts from fresh tissue were obtained by phenol: chloroform extraction and spin-column chromatography in Sephadex G-50 as described by Pappu *et al.* (1996). The sample consisted of about 1 $cm^2$ of leaf tissue or petioles or bark pulverized in liquid nitrogen before addition of 0.6 ml of extraction buffer (10% SDS, 1.0 M Tris pH8, 0.5 M EDTA) and an equal volume of phenol:chloroform (1:1 v/v). The samples were incubated at 70 °C for 5 min, then centrifuged 5 min at 14,000 rpm at room temperature. The supernatant was collected and a 17.5 μl aliquot used in a 50 μl RT-PCR reaction (10mM Tris HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100; 10 mM dithiothreitol; 2.5 mM $MgCl_2$; 0.1 M dNTPs and 100 pM of each primer). Occasionally, 25 μl reactions were performed by halving all the reaction component volumes. An alternate method to amplify both of the contiguous CP and p27 CTV genes in a single reaction was sometimes used. In this procedure, the CTV virions were first trapped onto microtiter plates and subsequently used for RT-PCR according to

Nolasco *et al.* (1993), with modifications. After performing the DAS-indirect ELISA, the plates (Immunolon 2 HB, Dynex Technologies, Chantilly, VA.) were washed twice each with PBS-T, then sterile distilled water and stored at -20 °C. For IC-RT-PCR, the RT reaction was performed in the plate wells by adding 45 µl of disruption buffer (10 mM Tris HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100; 10 mM dithiothreitol; 2.5 mM $MgCl_2$, 0.1 M dNTPs and 100 pM of each primer). The plates were then incubated at 70 °C for 5 min, then allowed to cool to room temperature. The following enzyme mixture was added: 10 U Avian Myeloblastosis virus reverse transcriptase (Promega, Madison, WI.), 2.5 U Taq polymerase (Promega), and 20 U RNasin (Promega) in a final volume of 5 µl, and the plates incubated at 42 °C for 45 min. The mixture was transferred to PCR tubes and amplified for 40 cycles at 90 °C, 50 °C, and 72 °C for 1 min each, and 10 min at 72 °C for the final extension. The specific primers used for the CP gene (CN119 and CN120) and the p27 gene (CN167 and CN168) are described in Table 2-1. When the CP and p27 genes were amplified together, primers CN119 and CN168 were used. The RT-PCR products were separated in agarose gels and photographed.

**Hybridization and detection by strain specific probes (SSP).** The CP-PCR products, after separation on an agarose gel, were blotted to a nylon membrane and fixed by UV cross-linking (UV Stratalinker 1800, Stratagene, La Jolla, CA). After pre-hybridization, 50 ng of the desired oligonucleotide probe (with a biotin label) were added, and the hybridization conducted for 1 hr at 37 °C with gentle agitation. After washing at the appropriate temperatures (50 °C for probe I, 58 °C for probe V, and 48 °C for probe VI and VII), the membrane was then incubated in 0.1 ml/$cm^2$ of streptavidin-horseradish peroxidase (SA-HRP) conjugate with gentle agitation for 45 min, rinsed and incubated with Super Signal CL-HRP, a chemiluminescent substrate (Pierce Inc., Rockford, IL). The reactions were then visualized on X-ray film. After each development, the probes were removed from the membrane with 0.4 N NaOH at 42 °C for 30 min. The same membrane was rehybridized successively with each of the oligonucleotide probes.

**Single-strand conformation polymorphism analysis (SSCP).** The performed SSCP analysis was similar to the protocol described by Febres *et al* (7) and Rubio *et al* (33). Depending on the DNA concentration after PCR, the p27 products were mixed with a denaturing solution (96% formamide, 20mM EDTA, 0.005% xylene cyanol and 0.005% bromophenol blue) in a final volume of 10 µl. The samples were denatured at 100 °C for 10 min, then chilled on ice for 2 to 5 min. Five-µl samples were loaded onto a 8% polyacrylamide gel (30:1 acrylamide: bis-acrylamide) prepared with TBE (53 mM Tris, 53 mM boric acid, 1.5 mM EDTA) and pre-cooled overnight at 4° C in a mini Protean II unit (Bio-rad. Laboratories, Inc., Hercules, CA). The gel was electrophoresed at 200 volts for 4 hr at room temperature using TBE as the running buffer. The unit was placed at 4 °C if the unit started to get too hot. After electrophoresis the gel was silver stained to visualize the gel bands.

## Results

**Indirect-DAS ELISA, IC-RT-PCR and RT-PCR.** Serological screening using G604 and MCA13 antibodies, for broad spectrum and severe strain detection of CTV, respectively, allowed the differentiation of the CTV isolates into two preliminary groups of either mild or severe isolates (Table 2-2). All isolates reacted with G604 indicating that all plants were infected with CTV. Mild isolates (101-3, 102-3, 158-3, 170-17, 175-

Table 2-1. Oligonuclotide primers used for CP and p27 RT-PCR amplification.

| Primer | Sequence | Gene | Product size |
|---|---|---|---|
| CN119 | 5'AGATCTACCATGGACGACGAAACAAAG3' (sense) | CP | 672 bp |
| CN120 | 5'GAATTCGCGGCCGCTCAACGTGTGTTAAATTTCC3' | CP | |
| CN167 | 5'CTATAAGTACTTACCCAAATC3' | p27 | 723 bp |
| CN168 | 5'AAGCTTCTAGAACCATGGCAGGTTATACAGTAC3' (sense) | p27 | |

12, 181-3, 194-3) were negative with MCA13 but positive with G604. The isolates 4-39, 7-26, 96-3a, 96-3b, 96-4b, and 161-30 were positive with both MCA13 and G604 indicating severe strains were present in the isolate. Seven isolates (93-17a, 96-3c, 96-3d, 96-9-6, 138-30, 163-30, 191-26) did not react with MCA13 suggesting they were mild or that the titer of severe strains, if present, was too low for detection at the time of the sampling. For all isolates tested, CP and p27 products were obtained by either IC-RT-PCR or/and RT-PCR, which enabled further testing with SSP and SSCP. The PCR products from all isolates reacted with the universal probe 0, confirming the presence of CTV. Ten isolates (93-17a, 96-9-6, 101-3, 102-3, 138-3, 158-3, 163-30, 175-12, 181-3, 194-3) reacted with mild strain probes VI and VII, but not with any other probes for stem pitting strains (probes II, III, IV, and V). Five isolates (7-26, 96-3c, 96-4b, 161-30, 191-26) reacted with probe I indicating the isolates contain decline strains of CTV. Furthermore, a third group of isolates hybridizing to probes I (decline probe) and VI and VII (mild probes) (96-3a, 96-3b, 96-3d, 170-17) suggesting a mixture of decline and mild strains present in the isolates. The isolate 4-39 reacted weakly with probe I, and strongly with probes V (stem pitting probe), VI and VII suggesting a mixture of decline, stem pitting, and mild strains present in the isolate. None of the isolates tested reacted with probes II, III, IV (Fig. 2-1, Table 2-2).

**SSCP patterns.** The SSCP patterns of the p27 PCR products of some of the isolates were compared with the patterns obtained for the Florida strains T36 (decline), T30 (mild), and isolate B249, a Venezuelan CTV isolate which causes stem pitting and which reacts with SSP probe V. Isolates 93-17a, 138-3, 163-3, 170-17, 175-12, 181-3 and 194-3 had SSCP patterns typical of T30 (mild strains). The decline isolates 161-3,

Table.2-2. Serological and molecular differences among some Florida isolates.

| ISOLATE | ELISA | | Biotin-labeled probes | | | | | | | | SSCP | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MCA13 | G604 | 0 | I | II | III | IV | V | VI | VII | p27 | Comments |
| 4-39 | 0.028 | 0.585 | + | +/- | - | - | - | + | +/- | + | n.a. | Mixture |
| 7-26 | 1.388 | 0.187 | + | + | - | - | - | - | - | - | n.a | QD |
| 93-17a | 0.000 | 0.092 | + | - | - | - | - | - | + | + | T30-like | Mild |
| 96-3a | 0.031 | 0.253 | + | + | - | - | - | - | + | + | T36-like | Mixture |
| 96-3b | 0.045 | 0.517 | + | + | - | - | - | - | + | + | T36-like | Mixture |
| 96-3c | 0.000 | 0.485 | + | + | - | - | - | - | - | - | T36-like | QD |
| 96-3d | 0.000 | 0.364 | + | + | - | - | - | - | + | + | T36-like | Mixture |
| 96-4b | 0.028 | 0.067 | + | + | - | - | - | - | - | - | n.a. | QD |
| 96-9-6 | 0.000 | 0.019 | + | - | - | - | - | - | + | + | T30-like | Mild |
| 101-3 | 0.044 | 0.396 | + | - | - | - | - | - | + | + | T30-like | Mild |
| 102-3 | 0.006 | 0.637 | + | - | - | - | - | - | + | + | T30-like | Mild |
| 138-30 | 0.000 | 0.260 | + | - | - | - | - | - | + | + | T30-like | Mild |
| 158-3 | 0.004 | 0.541 | + | - | - | - | - | - | + | + | n.a. | Mild |
| 161-30 | 1.654 | 0.133 | + | + | - | - | - | - | - | - | n.a. | QD |
| 163-30 | 0.000 | 0.124 | + | - | - | - | - | - | + | + | T30-like | Mild |
| 170-17 | 0.001 | 0.340 | + | +/- | - | - | - | - | + | + | T30-like | Mixture |
| 175-12 | 0.007 | 0.015 | + | - | - | - | - | - | + | + | T30-like | Mild |
| 181-3 | 0.005 | 0.518 | + | - | - | - | - | - | + | +/- | T30-like | Mild |
| 191-26 | 0.000 | 0.054 | + | + | - | - | - | - | - | - | T36-like | QD |
| 194-3 | 0.006 | 0.038 | + | - | - | - | - | - | + | + | T30-like | Mild |
| T30 | 0.007 | 0.211 | + | - | - | - | - | - | + | + | T30-like | Mild |
| T36 | 0.654 | 0.214 | + | + | - | - | - | - | - | - | T36-like | QD |
| Healthy | 0.003 | 0.003 | - | - | - | - | - | - | - | - | | |
| Blank | 0.001 | 0.001 | | | | | | | | | | |

+/-_= weak reaction. n.a.= data not available. QD= quick decline. Mixture= mixture of strains.

96-3c, 191-26, had SSCP patterns like T36, and isolates, which appear to be mixtures (96-3a, a different mild strain [Fig.2-2], 96-3b, 96-3d, and 101-3) also, had SSCP patterns like T30. Isolate 102-3 and 181-3 had SSCP patterns similar but not exactly like isolate T30, suggesting these isolates could be a different mild strain (Fig. 2-2).

## Discussion

Until recently, the selection of mild strains for use in MSCP was entirely an empirical process. In the past few years, we have been collecting apparently mild isolates of CTV for evaluation for MSCP with the desire to select additional mild strains for MSCP before *T. citricida*, present in Florida since November 1995, spreads CTV-D and CTV-SP strains to the point where the mild strains are no longer apparent in the field. Data from Australia (Broadbent, *et al.* 1996) and our experience, as reported here, suggests that it may take four sequential single aphid transmissions to obtain a "pure" strain of CTV.

The recent advances in the knowledge of the CTV genome (Pappu *et al.* 1994, Cevik 1995; Karasev *et al.* 1995; Cevik *et al* 1995) have resulted in the development of methods to differentiate CTV strains. In this study we have evaluated the recently developed methods of differentiation (serological differentiation of mild and decline CTV strains, PCR amplification of the coat protein gene followed by the SSP hybridization, and SSCP analysis of the p27 gene) to screen CTV isolates collected from surviving trees in areas severely affected by CTV decline on sour orange rootstock to determine if the isolates contain only mild, only severe, or mixtures of mild and severe strains. The evaluation for differentiation of CTV strains, as described in this paper, is applied for the detection of mild and severe strains from the original *in planta* culture as well as SAT sub-isolates.

The application of these methods of differentiation of CTV strains should enable

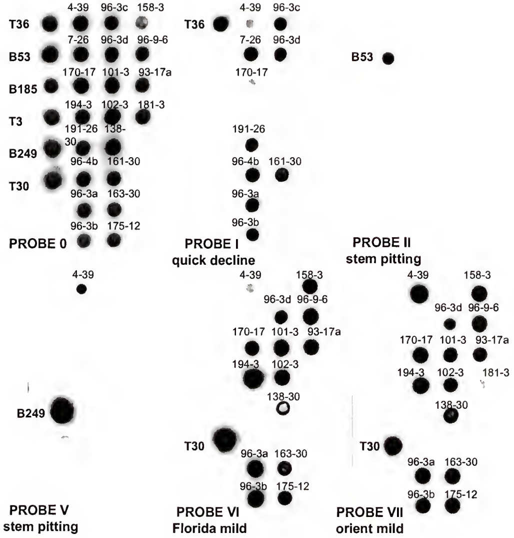


Fig. 2-1. Hybridization blots of Florida isolates with CP-CTV biotin-labeled probes. Probe 0, from a conserved CP region. Probe I, T36 and T66 (QD). Probe II, B1 and B53 (SP from Australia and Japan). Probe V, B128 and B249 (SP from Colombia and Venezuela). Probe VI, T26 and T30 (Florida mild). Probe VII, B188 and B215 (Orient mild).

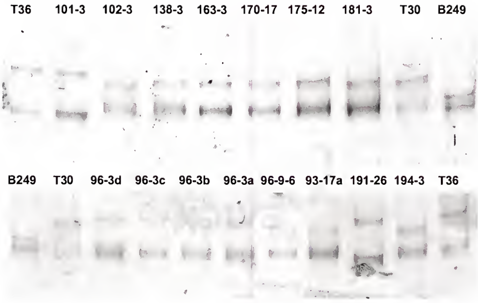


Fig. 2-2. SSCP patterns of some Florida CTV strains. Selected controls are T36 (Florida QD), T30( Florida Mild), B249 (Venezuela SP).

faster, and ultimately, better selection of mild strains, which may be useful for MSCP. It is interesting to note that of the nine isolates tested were collected directly from horticulturally superior appearing trees, one isolate (4-39) apparently was a mixture of mild, decline, and stem pitting strains; three isolates (96-3a, 96-3b, and 96-3d) apparently were mixtures of mild and decline strains; three isolates (7-26, 96-3c, 96-4b) apparently contained only decline strains, not mild; and only two isolates (93-17a and 96-9-6) appeared to be only mild strains. A previous study (Rocha-Peña 1990) suggests that isolates which give a high value in MCA13 ELISA, and moderate to high values in broad spectrum ELISA, such as isolates 7-26 and 161-30 (Table 1), probably do not contain isolates useful for MSCP. Isolates which give low to negative values in MCA13 ELISA, such as 4-39, 96-3a, 96-3b, 96-3d, may contain mild strains useful for MSCP. This may be due to the presence of mild strains which suppress the titer of the severe strains which may also be present. These results also confirm previous reports on the use of this methodology as a diagnostic strategy to predict biological activity of CTV isolates from samples collected in Florida, Morocco, Mexico, Portugal, Spain and Venezuela (Pappu *et al.* 1994; Akbulut 1996; Nickel *et al.* 1996; Niblett *et al.* 1997a; Niblett *et al.* 1997b).

Studies of CTV sub-isolates obtained as a result of SAT using *T. citricida* (Broadbent *et al.* 1996; Marais 2000) indicate that it is possible to segregate CTV strains from a source isolate. Eleven SAT sub-isolates were included in this study (isolate numbers greater than 100). Of these 11 isolates, only one (170-17) apparently is a mixture of mild and decline strains. Isolates 96-3c, and 191-26 did not react with MCA13 in ELISA, but reacted with SSP probe I, and their SSCP pattern were similar to T36, both tests indicating the presence of a decline strain of CTV.

## *CHAPTER 3*

## LOCALIZATION OF THE CP AND MINOR CP OF CTV IN RELATION TO THE GENOMIC RNA.

### Introduction.

Citrus tristeza virus (CTV) is an important member within the family *Closteroviridae* and is responsible for one the most devastating diseases of citrus worldwide. *Closteroviridae* constitutes a distinctive family among RNA viruses which includes several pathogens that affect major fruit and vegetables crops. CTV infects citrus, grapevine leaf roll virus (GLRaV) infects grapevine, apple stem grooving virus (ASGV) infects apple, lettuce infectious virus (LIV) infects lettuce and beet yellow virus (BYV) infects sugar beet, among others, causing important economic losses (Dolja, *et al.* 1994). In general, Closteroviruses show intermediate properties between the Sindbis-like and Corona-like viruses (Dawson 2000). Among the positive-strand RNA viruses, they have an unusually large genome, similar to the genomes of Coronaviruses and Toroviruses (Dolja *et al.* 1994). Other unique aspects of the Closterovirus morphology are a helical symmetry, flexuous particles with a characteristic thread-like structure (Bar-Joseph and Lee 1989). The closterovirus cytopathology is also quite characteristic, and CTV induces particular cytopathic effects which have been useful for diagnostic purposes (Schneider 1957a, 1957b, 1959; Brlansky *et al.* 1990; Ochoa *et al.*; 1994; Lin *et al.* 2000).

An interesting affinity with aphids, whiteflies and mealybug as vectors has been reported in the Closterovirus genus, and two levels of virus-vector specificity are noticeable for CTV and BYV. In fact, CTV is most efficiently transmitted by *Toxoptera citricida* among the eight aphid species which transmit CTV, whereas BYV is most efficiently transmitted by *Myzus persicae* as compared to the other 28 aphid species which transmit BYV (Dolja *et al.* 1994). The region of the CTV genome associated with aphid transmission is presently unknown.

Closteroviruses also contain a distinctive block of two capsid protein (CP) genes whose translation products encapsidate the genome in a polar fashion. The term “rattlesnake” structure has been proposed for this polar capping with the minor CP located at one end of the virion, and encapsidating about 5 percent of the virion (Agranovsky *et al.* 1995). Nevertheless, the term “polar structure” will be used in this study. It has been thought that the minor CPs of both BYV, the p24, and CTV, the p27, may play a role in aphid transmission (Agranovski *et al.* 1995; Febres *et al.* 1996). The minor CPs are thought to have evolved by duplication and subsequent divergence from the downstream CP gene (Boyko *et al.* 1992). Direct and indirect evidence for the CTV polar structure have been reported. It was demonstrated that only one terminus of the CTV virion was encapsidated by the p27 minor CP by using SSEM and gold-labeled antibody prepared to bacterial expressed p27 (Febres *et al.* 1994). The p27 of CTV encapsidates a segment approximately 75 to 85 nm long, in concordance with the corresponding 75 nm segment encapsidated by the p24 reported for BYV (Agranovsky *et al.* 1995; Febres *et al.* 1996). Additional observations indicated the presence of shorter particles which also contained the p27 CP, which suggested the possible *in planta* polar encapsidation of defective RNA (D-RNA) (Mawassi *et al.* 1995a, 1995b,1995c) and/or subgenomic RNAs (Febres *et al.* 1996).

Since a better understanding of CTV encapsidation mechanism is required, this study determines the position of the polar structure on the CTV genome.

## Materials and Methods

**Virus isolates and host.** The Florida decline strain, T36, also designated as isolate B2 in the Collection of Exotic Citrus Diseases, in Beltsville, MD, USA, was maintained *in planta* in Mexican limes (*Citrus aurantifolia* (Chrismt.) Swing.) and used as the permanent source of infected tissue for CTV purification and immuno-capture reverse transcriptase- PCR (IC-RT-PCR) throughout this study. The plants were kept in a partially shaded, air-cooled greenhouse with temperatures ranging from 21 to 30°C at the University of Florida's Citrus Research and Education Center, Lake Alfred.

**Virus purification.** CTV particles were purified following a modified procedure similar to others previously reported ( Bar-Joseph *et al.* 1985; Lee *et al.* 1987, Garnsey *et al.* 1981a, Garnsey *et al.* 1981b). Lots of 40 g of fresh young bark tissue from infected and healthy plants were peeled and frozed at –80 °C until used. The frozen tissue was then pulverized in liquid nitrogen, and mixed with 160 ml of 0.05 M Tris buffer, pH 8, containing 0.1% of 2-Mercaptoethanol, and filtered through four layers of buffer-wet sterile cheesecloth. The filtered homogenate was centrifuged at 11,325 g for 10 min at approximately 4-10 °C in a Beckman JA10 rotor, then 1.6 ml of 20% Triton X-100 was added to the supernatant. The supernatant was then layered on top of a step gradient prepared by layering 5 ml of 25% sucrose on top of 5 ml of 60% sucrose made using in 0.05 M Tris, pH 8. The sucrose step gradient was centrifuged at 65,095 g for 15 h at 4 °C in a SW28 rotor. Following centrifugation, 1.5 ml fractions were removed from the bottom using a large 22G 3½ inch long spinal tap needle and 10 ml syringe. The bottom fractions were discarded and the second and third 1.5 ml fractions pooled from all six centrifuge tubes, labeled as fractions 2 and 3, respectively, and dialyzed in 2 liters 0.05 M Tris, pH 8 (2 changes/4 hr each). The dialysate was centrifuged at 7,300 g for 10 min at 4 °C in a JA20 rotor. The volume of the recovered supernatant was adjusted to 28 ml using 0.05 M Tris, pH 8, and mixed with 12 ml of 3.0 molal $Cs_2SO_4$ in 0.05 M Tris, pH 8.0, and centrifuged at 209,500 g for 20 hr at 4 °C in a VTi50 rotor. The visual observation and collection of bands was enhanced by polarized light illumination. Each pooled fraction from the sucrose step gradient (labeled as fraction 2 and 3) contained top (T) and bottom (B) bands following isopycnic centrifugation (Fig. 3-1). The top bands were collected using a syringe as described before and labeled 2T and 3T. The lower bands, labeled 2B and 3B, were also collected. The CTV yields were monitored by ELISA and IC-RT-PCR.

**Sonication and fractionation by rate zonal centrifugation.** Fractions 2B and 3B from the isopycnic gradients were sonicated 15 sec. at 20 watts/cm$^2$ in a sonicator model W-225 R (Heats Systems-Ultrasonics Inc., Plainview, L.I., N.Y.). The viral preparations were size fractionated by layering c.a. 3.5 ml of the sonicated preparation onto a 10-40 % rate zonal centrifugation (RZC) sucrose gradient made by overlayering 1.5 ml each of 10, 15, 20, 25, 30, 35, and 40 % sucrose made in 0.05 M Tris, pH 8 (Garnsey *et al.* 1981a, Garnsey *et al.* 1981b). The gradient was centrifuged 3 hr at 236,500 g at 4 °C in a SW41 rotor. After centrifugation, the gradient was fractionated using an ISCO Model 640 fractionator and 0.4 ml fractions were collected. Fractionation was done at 4 °C, and the fractions were handled on ice.

**DAS-Indirect ELISA.** The relative virus concentration in each fraction was estimated by ELISA performed as previously described (Bar-Joseph *et al.* 1979b; Garnsey and Cambra 1991). The rabbit polyclonal antiserum CREC 31 was used at a 1:1000 dilution from a 1:1 glycerol stock. CREC 31 antiserum was prepared against a whole, unfixed purified virus preparation of a mixture of several isolates from the Collection of Exotic Citrus Disease, maintained at Beltsville, MD, (Manjunath 1996). CREC 31 reacts against both p25 and p27 CPs and recognizes mainly p25. The polyclonal antiserum UF 34 was used at a 1:500 from a 1:1 glycerol stock. UF 34 was prepared in a rabbit against a bacterial expressed p27 antigen, and specifically recognizes only p27 (Febres *et al.* 1994). Five µl of each purified CTV fraction were mixed with 95 µl of 0.05 M Tris, pH 8, previously loaded in the wells of the ELISA microtiter plate. A mixture of monoclonals 3DF1 and 3CA5 (0.05 µg/ml each) were used as the secondary antisera; rabbit antimouse alkaline phosphatase conjugate (Sigma) was used for detection.

**IC-RT-PCR.** The IC-RT-PCR was performed according to Nolasco *et al* (1993) with modifications after the DAS-indirect ELISA. For IC-RT-PCR, the RT reactions were performed in antibody-coated PCR tubes using either CREC 31 or the UF 34 p27-specific, both diluted 1:1000 from 1:1 glycerol stocks. Twenty five µl of each fraction from the RZC-sucrose gradient diluted in equal volumes of 0.05 M Tris, pH 8, were incubated overnight at 4 °C. The liquid was discarded and the plates washed with PBS-triton-sucrose (0.02% Triton and 5% sucrose) three times. Then 22.5 µl of virus disruption buffer (10 mM Tris HCl, pH 9.0; 50 mM KCl, 0.1% Triton X-100, 10 mM dithiothreitol (DTT), 2.5 mM $MgCl_2$, 10 mM dNTPs and 5 mM of each primer for the amplification of the 5'- and 3'- termini, Table 3-1) were added. The tubes were incubated

Table. 3-1. Oligonucleotide primers used for the amplification of 5’-and 3’- termini of citrus tristeza virus by IC-RT-PCR using UF 34 and CREC 31 antisera.

| Primer | Sequence | Position |
|---|---|---|
| C 121 | 5’AATTTCACAAATTCAACCTGTTCG3’ (sense) | 1-510 nt |
| C 335 | 5’CCGCTTTGCCTGACGGAGGGACCTCT3’ | |
| C 193 | 5’GAACCCTAGGTGATAATACTAGCGGACAAACT3’ (sense) | 18,392-19,292 nt |
| C 173 | 5’CATGCCATGGTGGACCTATGTTGGCCCC3’ | |

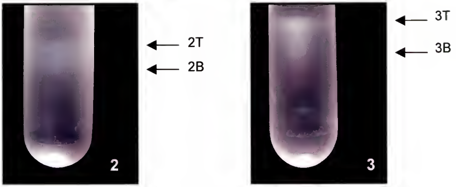


Fig. 3-1. CTV purified bands after $Cs_2SO_4$ isopycnic gradient centrifugation. A) Bands 2T and 2B from sucrose step gradient fraction number 2. B) Bands 3T and 3B from sucrose gradient fraction number 3.

incubated at 70 °C for 5 min, then allowed to cool to room temperature. The following enzyme mixture was added: 50 U Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI.), and 20 U RNasin (Promega) in a final volume of 2.25 μl, followed by a cDNA incubation reaction of 1 h at 42 °C. Taq polymerase (Promega), 1.25 U, was added before the PCR was started. The IC-RT mixture was PCR amplified for (1) 95 °C for 20 sec; (2) 30 cycles at 95 °C for 20 sec, 50 °C and 72 °C for 1 min each, and (3) 10 min at 72 °C for the final extension. Each IC-RT-PCR was conducted separately for both the 5'- and the 3'-terminus, and the two terminus products of each RZC sucrose gradient were then loaded together into the same gel lane, then separated by electrophoresis in a 1.2 % agarose gel before visualization and photography.

**RNA extraction and Northern blot hybridization analysis.** The dsRNA extracts from infected host tissue were prepared according to Albiach-Martí (unpublished). Total RNA was extracted from 300 μl of RZC sucrose gradient fraction by using the phenol:chloroform procedure described by Rosner *et al.* (1983). Agarose gel electrophoresis and Northern blot hybridization were performed as reported by Lewandowsky and Dawson (1998). The membrane was probed with anti-digoxigenin-alkaline phosphatase Fab fragment and developed with CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxe-tane-3,2'-(5'-chloro)tricyclo[3.3.1.1$^{3.7}$]decan}-4-yl)phenyl phosphate) chemiluminescent substrate following the manufacturer's directions (Roche Molecular biochemicals. Indianapolis. IN) The terminal probe corresponded to 640 nt of the 5'- end.

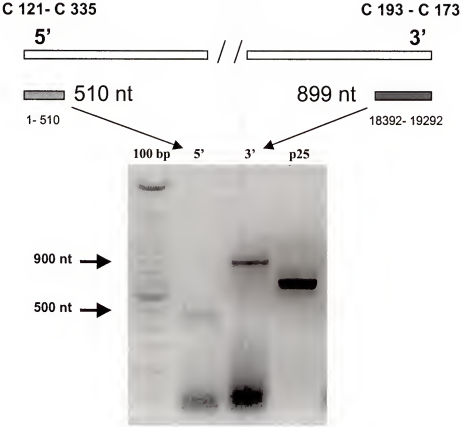


Fig. 3-2. Strategy used for the IC-RT-PCR amplification of 5'and 3' termini by specific primers. Lane on left, 100 bp ladder; lane center-left, the 510 nt 5'- terminus product; lane center-right, the 899 nt 3'- terminus product; lane right, the 672 nt of p25 CP product as control. For primer sequences refer to Table 2-1 and Table 3-1.

## Results

**IC-RT-PCR conditions and virus purification.** The IC-RT-PCR conditions were optimized for the amplification of a 5'- terminus product of the first 510 nt (primers C 121 and C 335) and a 3'- terminus segment of 899 nt (primers C 193 and C 173) (Fig 3-1). During the purification steps, CTV consistently migrated as a broad band in the lower 1/3 of the 25-60 % sucrose step gradient, resulting in the collection of two fractions (labeled as 2 and 3). Each of those aliquots were subsequently further purified into two bands in a $Cs_2SO_4$ isopycnic gradient resulting in a total of four fractions 2B, 2T, 3B and 3T (Fig. 3-1). The serological reaction (ELISA) of bands 2B and 3B from the isopycnic gradients was stronger than that of bands 2T and 3T (data not shown), and it was not possible to amplify the 5'-, nor the 3'- nor the p25 gene by IC-RT-PCR using either CREC 31 or UF 34 antiserum from the 2T or 3T bands. This suggests that the 2T and 3T bands from the isopycnic gradients contain broken CP or empty CTV fragments, (Fig. 3-3). However, the 5'-and 3'- termini were both amplified from both the 2B and 3B bands obtained in the same isopycnic gradients, but 2B, which originated from the lower fraction in the sucrose step gradient, allowed a better amplification of the 5'- terminus. The 2B and 3B bands from the isopycnic gradients were further examined by centrifugation on RZC sucrose gradients. The presence of CTV was estimated by ELISA and IC-RT-PCR from each fraction collected from the 10-40% RZC gradients using CREC 31 antiserum to trap p25 and UF34 antiserum to trap p27 in separate tubes. In general, ELISA with CREC 31-p25 trapping yielded higher O.D. values at 405 nm than the UF 34-p27 trapping. This would be expected if the p25 encapsidates most of the virion and therefore, is more abundant (Fig. 3-4).

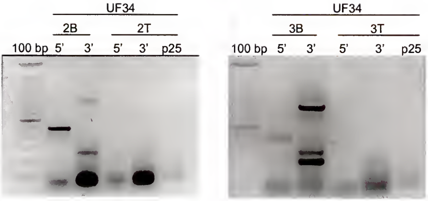


Fig. 3-3. IC-RT-PCR, using UF 34 antisera specific for the p27 minor capsid protein (CP) from bands generated after $Cs_2SO_4$ isopycnic gradient centrifugation. Note that the 5'-, and 3'-termini, and p25 major CP were not amplified from bands 2T and

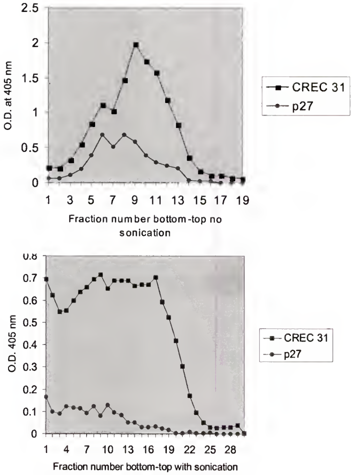


Fig. 3-4. CTV distribution pattern through a linear 10-40 % rate zonal centrifugation (RZC) sucrose gradient as determined by ELISA. CTV reactivity was estimated by ELISA using UF 34 antiserum, specific for p27 minor coat protein (CP) as trapping antiserum (1:500 from 1:1 glycerol stock) and 3DF1+3CA5 (0.05 μg/ml each) as secondary antibody. CREC 31, made against purified CTV preparations and which recognizes both CPs p25 and p27 (1:1000 from 1:1 glycerol stock), was used as control to estimate total amount of virus. Top: without sonication of band 2B. Bottom: after sonication of band 2B. Fraction 1 is at the bottom of the RZC.

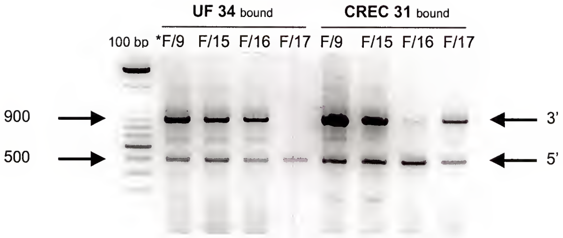


Fig. 3-5. Specific amplification of the 5'-terminus of CTV RNA by IC-RT-PCR of 10-40 % RZC-sucrose gradient fractions with specific antisera UF 34 specific to p27 and CREC 31, which recognizes both p25 and p27. * F/ refers to fraction number from RZC sucrose gradients, refer to Fig. 3-4.

**Specific IC-RT-PCR of only one terminus.** A different migration pattern through the RZC sucrose gradient was generated after the sonication of the virus in the 2B and 3B bands. Experiments with and without sonication were repeated five times. When the UF 34 antiserum, specific to p27, was used for immunocapturing, the 5'-terminus of CTV was consistently amplified from all fractions throughout the RZC sucrose gradient which had ELISA activity for CTV. However, the 3'-terminus was not amplified in fractions near the top of the gradient when using CREC 31 for trapping, despite that ELISA indicated CTV was present (Fig.3-5). It is noticeable that both the 5'- and 3'- termini were amplified along the 10-40 % RZC sucrose gradient when IC-RT-PCR was performed trapping with the control polyclonal CREC 31, made against whole CTV (Fig.3-5).

**Examination of D-RNAs.** The dsRNA extracts from different T36 infected Mexican lime donors, and total RNA extractions using phenol:chloroform from RZC sucrose gradient fractions were probed with a 5'-termini specific riboprobe after gel electrophoresis and Northern blotting. A diverse size population of naturally occurring D-RNAs was detected in the donor plants. Moreover, the D-RNAs were detected in the RZC sucrose gradient fractions, indicating that a mixture of virions and D-RNAs were present in all sonicated fractions (Fig. 3-6)

## Discussion

Purified CTV virions were examined to determine which end of the RNA genome of CTV is encapsidated by the p27 minor CP. This was approached by specifically immunocapturing one terminus with an antisera specific for the CTV p27 minor CP.

CTV consists of large flexuous virions which may be fractionated by size via centrifugation through a 10-40 % RZC sucrose gradient. Complete virions and larger fragments of the virions migrate faster and are nearer the bottom part of the gradient with progressively smaller fragments in fractions nearer the top. Using IC-RT-PCR and trapping with UF 34 specific for the minor p27 CP, the 5'-terminus of CTV RNA always was amplified from all fractions which contained CTV as detected by ELISA when CREC 31, made against purified CTV, was used for trapping. However, the 3'- terminus was not amplified from fractions near the top of the gradients where IC-RT-PCR trapping with p27 specific antiserum, gave only a 5' termini product. Thus, near the top of the gradient, only the small CTV fragments containing p27 at one end were captured by the p27 antiserum. The bottom and middle fractions of the RZC gradients contained complete particles and also probably D-RNAs carrying the two termini allowing the 5' and 3' amplification. D-RNAs with a 3' termini between nucleotides 18297 and 19226 have been reported for the Israel VT strain (Bar-Joseph *et al.* 1997). A similar result has been reported for BYV (Zinovking *et al.*1999).

Analysis of dsRNA and of total RNA extracts from RZC sucrose gradients from different infected donor plants by a Northern blot hybridization using a 5'- specific riboprobe revealed the presence of a naturally occurring population of D-RNAs of different sizes as well as the presence of CTV genomic and subgenomic RNAs. This result explains the wide range of CTV fragments detected by ELISA after the RZC sucrose gradients with or without sonication. Also, the amplification of 5'- and 3'-termini from numerous RZC fractions, and not near the top, where small CTV fragments with and without p27 allow the specific 5'- discrimination by UF34 antiserum.

It has been speculated that the p27 might be like a RNA-less shell retained only by protein-protein interaction as conjetured for the BYV p24 (Agranovski *et al.* 1995). Previous report suggesting that the minor CPs of CTV and BYV are prone to degradation (Agranovski 1995, Agranovski *et al.* 1995; Bar-Joseph *et al.* 1997; Zinovkin *et al.* 1999) might now be revisited. This study demonstrates that the p27 is not a fragile protein structure since virions are manipulated through several purification steps and can still be immunocaptured and the 5'-terminus amplified.

Recently, the formation of viable virions was studied using deletion mutants generated from a wild type infectious CTV clone. It was demonstrated that both CP genes are essential for efficient formation of viable virions (Satyanarayana *et al.* 2000). Thus one functional role of p27 has been demonstrated, but it is still possible that the minor CP might be involved in other roles. For example, the p27 has been detected in the cell walls, the soluble and the membrane fractions of CTV infected cells ( Febres *et al.* 1994). The homologous BYV p24 minor CP has been found predominantly in the soluble fraction (Agranovsky 1995).

The location of p27 at the 5'- terminus suggests a possible signaling that could target the virion through membranes or plasmodesmata during an early cell to cell movement. Furthermore, an interaction of p27 with some other vector-element(s) that might facilitate the vector transmission has been also proposed (Agranovski 1995; Agranovski *et al.* 1995, 1995b; Febres *et al.* 1996). This implies the existence of vector-encoded determinants that interact with viral determinants for cooperation during virus transmission. The minor amount of p27, encapsidating about 5 percent of the genome, as compared to p25, which encapsidates 95 percent, argues in favor of the p25 CP, rather

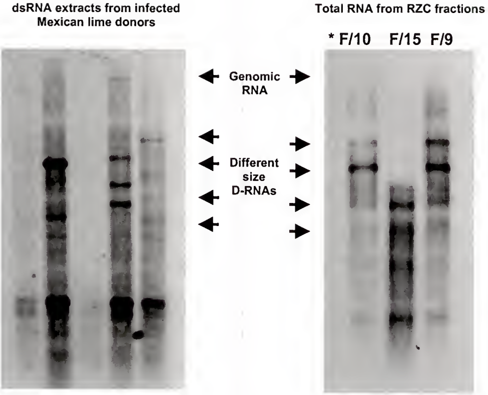


Fig. 3-6. CTV defective RNAs (D-RNAs) detected by Northern blot hybridization using a 5'- specific riboprobe Left: analysis of the dsRNA extracts from different Mexican limes plants infected with CTV isolate T36. Right: analysis of total RNA extracted from fractions collected from a rate zonal centrifugation sucrose gradient following sonication. *F/ indicates fraction number from a RZC sucrose gradient; refer to Fig. 3-4.

than the minor p27, for the major role in transmission interactions. Other proteins produced by CTV, or the aphid as well, also could be involved. Further research in the field of the host-virus and vector-virus interaction will be necessary to determinate additional functions and the precise protein-protein interactions.

## *CHAPTER 4*
# STUDIES OF HETEROLOGOUS ENCAPSIDATION IN TRANSFORMED PLANTS EXPRESSING THE CAPSID PROTEIN OF CITRUS TRISTEZA VIRUS.

## Introduction.

The first plant-virus interaction and the subsequent early events of virus replication are poorly understood. Once a virus enters a cell, the protective capsid protein (CP) is necessarily removed, and its viral nucleic acid is exposed for translation. Then the viral replication follows. During this process, the viral polymerase, in association with host proteins, produces copies of the viral genome (Strauss and Strauss 1999). The mechanism of replication varies, depending on the type of nucleic acid, the sense, and whether the viral genome is double- or single-stranded. Ultimately, newly formed virus particles are assembled from proteins and nucleic acids originated during the replication, then the newly assembled particles spread, primarily moving cell to cell, to infect the whole plant (Strauss and Strauss 1999; Gilbertson and Lucas 1996).

Under natural conditions, multiple infections with different virus species or different strains of the same virus in a single host are commonly observed (Falk and Bruening 1994). The replication and assembly of co-infecting viruses might occur simultaneously in close proximity within the cell. Occasionally, the nucleic acid of one virus may be encapsidated with the coat protein (CP) of another virus. The term "heterologous encapsidation" (Kassanis 1961) has been used to describe this phenomenon. Two other terms have been applied to better describe two specific cases of heterologous encapsidation: "Phenotypic mixing" which describes the heterologous encapsidation of the nucleic acid of one virus with the capsid protein from other viruses involved in the mixed infection; "Transcapsidation" which describes the fully heterologous encapsidation of the nucleic acid of one virus with the capsid protein of another virus or strain, resulting in a type of genomic masking (Farinelli, *et al.* 1992).

Heterologous encapsidation was first reported by Kassanis (1961), and experimentally confirmed to occur with plant viruses by Rochow (1970). It was demonstrated by serological inactivation experiments in which part of the genomic RNA of Barley yellow dwarf luterovirus (BYDV) MAV isolate was encapsidated with BYDV RPV isolate CP. Moreover, the transmission of the BYDV MAV isolate is dependent on the presence of the BYDV RPV CP since only heterologous encapsidated particles with the BYDV RPV CP could be transmitted by the aphid vector, *Rhopalosiphum padi*. The heterologous encapsidation could be visualized directly by electron microscopy using antibodies specific to CPs of each strain (Bourdin and Lecoq 1991; Farinelli *et al.* 1992). Additionally, detection of heterologous encapsidation could be enhanced by immunohybridization assays which allow the analyses of both the CP and the nucleic acid (Creamer and Falk, 1990). Heterologous encapsidation has been reported for viruses belonging to different families (Hordeivirus, Bromovirus, and Tobamovirus), but the phenomenon is not expected to occur among virus families having different architecture (Kaper and Geelen 1971; Peterson and Brakke 1973 ; Dodds and Hamilton 1974). More recent experimentation with potyviruses and furoviruses indicated that heterologous encapsidation is only possible between viruses within the same family or from the same species (Maiss *et al.* 1994).

The potential biological impact of heterologous encapsidation in the field has been controversial. Fuchs and Gonsalves (1998) indicated there was no natural spread of cucumber mosaic virus (CMV) in field trials where plants expressing the CP of a highly aphid-transmissible strain of CMV were inoculated with a non-aphid transmissible strain of CMV. Healthy non-transgenic controls did not become infected. However, de Zoeten (1991), Robinson (1996), and Korte *et al.* (1995) argue that CP transgenic plants must be carefully assessed before release since the risk of heterologous encapsidation and recombination could lead to the formation of new viral variants. Until now, there has been a general consensus about the involvement of heterologous encapsidation with aphid transmission, since it has been demonstrated that heterologous encapsidation enables aphid transmission of non-transmissible viruses at least with BYDV.

The mild strain cross protection phenomenon has been hypothetically associated with the capture of invading nucleic acid of the second virus by the CP of the first infective strain of tobacco mosaic virus (TMV) replicating in the same cellular niche (de Zoeten and Fulton 1975). While this theoretical principle of heterologous encapsidation might explain the mechanism of viral cross protection, experimentation with defective strains of TMV argue against the involvement of virus CP in cross protection (Zaitlin 1976). More recently, a cytoplasmic post-transcriptional mechanism, or gene silencing, which prevents virus replication has been demonstrated by English *et al.* (1996) and has been associated with the cross protection mechanism. According to this theory, multiple copies of subgenomic-RNAs (sgRNAs) or defective RNAs (D-RNAs) regulate the replication of the second virus.

Citrus tristeza virus (CTV) occurs in mixtures of strains in isolates (Bar-Joseph *et al.* 1989; Bar-Joseph and Lee 1989; Moreno *et al.* 1991; Cevik 1995 ; Cevik *et al.* 1996; Febres *et al.* 1995; Rubio *et al.*1996; Genc, 1998; Broadbent *et al.* 1996). Heterologous encapsidation could play a role as a non-genetic mechanism to enhance the CP variability of a CTV population. The exchange of CP elements required for aphid transmission could improve transmission and perpetuation. Plant transformation with various CP genes from different CTV strains has been addressed as a possible mechanism for obtainingCTV resistance. This study focuses on the heterologous encapsidation in transgenic plants expressing CTV CP which may occur with subsequently inoculated strains of CTV.

## Material and Methods

**Examining the heterologous encapsidation of CTV in transformed plants expressing the T36 coat protein sequence.** To examine whether heterologous encapsidation occurs between CTV CP expressed by transformed citrus and CTV virions, two mild isolates of CTV, T30a and T11a, and one decline isolate of CTV, T36, were graft-inoculated into transformed sour orange plants which express the major T36 CP gene (Gutiérrez *et al.* 1997). Transformed plants without inoculation, and non-transformed plants were also included as controls. The virus was allowed to replicate for at least a month, and testing was then conducted on new flushes induced after pruning. Each CTV isolate was inoculated into thirteen transformed plants, and a total of 104 plants from the same source were analyzed in this experiment. Each plant was considered an experimental unit.

**Plant source.** The micro-grafting technique developed by Skaria (1997), was the propagation method used for the production of sour orange (*Citrus aurantium* L.) grafted onto sour orange (*Citrus aurantium* L.) and transformed sour orange grafted onto sour orange plants. The transgenic sour orange expressed the CTV T36 CP gene as previously reported (Gutiérrez *et al.* 1997). The sour orange bud source was tested by DAS-ELISA using CREC 31 as coating antisera and G604 as secondary antibody before micro-grafting and found to be CTV free. Sour orange liners were grown in Deepots$^{TM}$ containers (Stuewe & Sons, Inc., Corvallis, Oregon), and the 4-P soil mixture from Fafard, Agawan, MA, was used. Fertilization was according to Roistacher (1991). The micrografted plants were mantained at 21-27 °C for four weeks and then transferred to a greenhouse maintained at 24-32.°C during the test.

**CTV isolates.** Source plants for the isolates used in this study are maintained as *in planta* cultures in Madame Vinous sweet orange at the University of Florida's Citrus Research and Education Center, Lake Alfred. The isolate T36, also labeled as the B-3 accession in the Collection of Exotic Citrus Diseases, Beltsville, MD. USA, is a decline strain; T30a (B2 accession in the Beltsville collection) has been characterize as mild strain (Rosner *et al.* 1986). Both T36 and T30a are naturally occurring isolates from Florida and have been sequenced and characterized at the biological, serological and molecular levels (Permar *et al.* 1990; Rocha-Peña *et al.* 1991, 1992; Karasev *et al.* 1995; Vives *et al.* 1999; Albiach-Martí *et al.* 2000). The third isolate, T11a, induces very mild symptoms with little or no stunting on Mexican lime (*C.* *aurantifolia* (Chrismt.) Swing.), and lacks cross-protection ability (Rocha-Peña *et al.* 1995; Yokomi *et al.* 1987). It does not react with the monoclonal antibody MCA-13 (Permar et al. 1990), and reacts with probes 0 (universal probe) and probe VI (Florida mild probe) by strain specific probe hybridization (Cevik, personal communication). All inoculations were made by grafting.

**DAS-Indirect ELISA and direct tissue blot immunoassay (DTBIA).** Fresh, tender bark tissue samples were pulverized in liquid nitrogen and stored at -80 °C until analysed. Double antibody sandwich (DAS)-indirect ELISA assays were performed as previously described (Bar-Joseph *et al.*1979b; Garnsey and Cambra, 1991). The rabbit polyclonal UF34 antiserum (Febres *et al.* 1994) also was used at a 1:500 dilution from a 1:1 glycerol stock to immunotrap virions by the p27 (minor CP). UF34 was prepared against a bacterial expressed p27 minor CP-antigen and specifically recognizes only the p27 CP of T36. Goat polyclonal antibody, G604, was used at 1:500,000 from a 1:1 glycerol stock. The G604 was made against a bacterially expressed p25 CP from the Indian isolate of CTV B227, (R. Lee and K. L. Manjunath, unpublished) and was used for broad spectrum detection of CTV. The monoclonal antibody, MCA 13, used at a 1:20,000 dilution (Permar *et al.* 1990) was used for selective detection of severe CTV strains. Anti-goat and anti-mouse antibody-alkaline phosphatase conjugates (Sigma, St. Louis, MO) were used as appropriate for detection. $OD_{405\ nm}$ values three times that of the healthy controls were considered to be positive for CTV. ELISA plates were stored at –20 C for later use with inmuno capture reverse-transcriptase-PCR (IC-RT-PCR).

Direct tissue blot immuno assay (DTBIA) was performed according to Garnsey *et al.* (1993) on all experimental transformed plants before inoculation with T30a, T11a and T36 infected tissue to ensure their expression of the T36 CP. MCA 13 was used at a dilution of 1:20,000.

**IC-RT-PCR.** In this procedure, the CTV virions were first trapped onto microtiter plates and subsequently used for RT-PCR according to Nolasco *et al.* (1993), with modifications. After performing the DAS-indirect ELISA, the plates (Immunlon 2 HB, Dynex Technologies, Chantilly, VA.) were washed twice with PBS-T, then with sterile distilled water and stored dry at -20 °C. For IC-RT-PCR, the RT reaction was performed in the plate wells by adding 45 µl of disruption buffer (10 mM Tris HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100; 10 mM dithiothreitol; 2.5 mM $MgCl_2$, 0.1 M dNTPs and 100 pM of each primer). The plates were then incubated at 70 °C for 5 min, then allowed to cool to room temperature. The following enzyme mixture was added: 10 U Avian Myeloblastosis virus reverse transcriptase (Promega, Madison, WI.), 2.5 U Taq polymerase (Promega), and 20 U RNasin (Promega) in a final volume of 5 µl, and the plates incubated at 42 °C for 45 min. The mixture was transferred to PCR tubes and amplified for 40 cycles at 90 °C, 50 °C, 72 °C for 1 min each, and 10 min at 72 °C for the final extension. The specific primers used for the CP gene (CN119 and CN120) are described in Table 2-1, (Chapter 2). The RT-PCR products were separated by electrophoresis in agarose gels and photographed over an ultraviolet transilluminator with a short wave UV light.

**Hybridization and detection by strain specific probes (SSP).** The SSP procedure was performed according to Cevik *et al.* (1993) The CP-PCR products, after separation on an agarose gel, were blotted to a nylon membrane and fixed by UV cross-linking (UV Stratalinker 1800, Stratagene, La Jolla, CA). After pre-hybridization, 50 ng of the desired oligonucleotide probe (with a biotin label) was added, and the hybridization conducted for 1 hr at 37 °C with gentle agitation. After washing at the appropriate temperatures (50 °C for probe I (decline probe), and 48 °C for probe VI (Florida mild probe), the membrane was then incubated in 0.1 ml/cm$^2$ of streptavidin-horseradish peroxidase (SA-HRP) conjugate with gentle agitation for 45 min. The membrane was then rinsed and incubated with Super Signal, a chemiluminescent-horse radish peroxidase (CL-HRP) substrate (Pierce Inc., Rockford, IL). The reactions were then visualized on X-ray film. After each development, the probes were stripped from the membrane with 0.4 N NaOH at 42 °C for 30 min. The same membrane was rehybridized sequentially with each of the oligonucleotide probes.

## Results

**CTV T36-CP expression in transformed citrus plants.** The T36 CP expression was confirmed in all transformed sour orange host by DTBIA. The CTV T36 CP location in phloem tissues was determined using the severe strain-selective monoclonal MCA13 and using the polyclonal goat G604 as a control. An intensely stained phloem area was easily distinguished in non-transformed plants infected with T36 and in transformed plants which were not inoculated, but plants infected with T36 virions showed a more intense staining than the transformed sour orange plants. A faint green image or very light pink background was observed in samples of plants inoculated with T30a, T11a, and in healthy plants used as controls (Fig. 4-1. A). The results indicated T36 CP was actively expressed in the phloem in transformed plants as reported by Gutierrez *et al.* 1997. Moreover, following after total nucleic acid extraction the T36 CP gene products were amplified by PCR using primers for the T36 CP gene (CN119 and CN120) (Table 2-1) (Fig. 4-1 B) indicating that the T36 CP gene, indeed, was incorporated into the transformed sour orange plants under study.

**Trapping CTV isolates with UF 34.** The UF 34 antiserum was produced against a bacterial expressed antigen in a rabbit, and the polyclonal antiserum is specific for the minor CP, p27, of CTV (Febres *et al.* 1994). UF 34 showed a high affinity for the homologous T36 minor CP, it also recognized the minor CPs of T30a, T68, and T11a, but with a lower affinity (Table 4-1; Fig. 4-2, Fig. 4-3). However, trapping of T11a is higher than for T30a and T68. A dilution experiment using UF 34 as the coating antiserum with 1:1000 to 1:35,000 dilutions made from a 1:1 glycerol stock was evaluated using T36 and T30a (1:10) as antigens (Fig. 4-2). UF 34 immuno captured the p27 CP of T36 with higher affinity than T30a, T68 and T11a as observed in Fig. 4-3.

**DAS-Indirect ELISA and DIBA.** The presence of CTV was detected by trapping the virions by the minor CP using UF 34, specific for the p27, as coating antibody and either G604 as the secondary antiserum for broad spectrum detection of all virions, or by MCA 13 as the secondary, which would detect severe CTV (T36) only. As the transformed plant express p25, the major CP, this should allow for trapping of the virions transformed plants which express the T36 CP and which were inoculated with T11a reacted positively against the severe strain specific monoclonal, MCA 13. No reactivity was observed among the thirteen transformed plants inoculated with T30a. All transgenic plants inoculated with T36 reacted positively against the MCA 13 and G604 secondary antibodies.

**Hybridization and detection by strain specific probes (SSP).** The virions from T11a inoculated plants which were immuno-captured by the p27 and which reacted positively against MCA 13 were subjected to RT-PCR amplification. T30a and T36 virions were used as controls. The two T11a samples were found to hybridize with probe

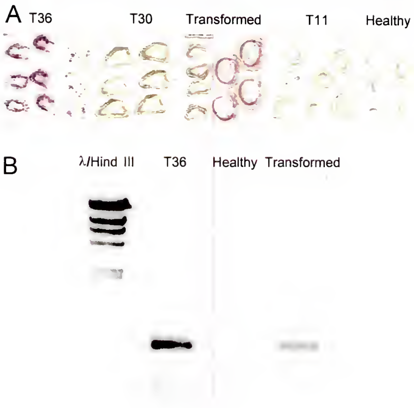


Fig. 4-1. Expression of CTV T36 isolate coat protein (CP) in transformed sour orange plants. A. DTBIA impressions developed using MCA 13 (1:20,000), note a purple stain on T36 and the transformed citrus. T36= sour orange inoculated with T36; T30a= sour orange inoculated with T30a; Transformed= transformed sour orange, T11a= sour orange inoculated with T11a; Healthy = sour orange without inoculation. B. RT-PCR and PCR amplification of the T36 CP gene from infected and transformed sour orange plants using primers CN119 and CN 120. Lane on left contains a standard of λ DNA digested by Hind III; lane 2 contains the T36 CP gene product amplified from a sour orange plant infected with T36; lane 3 contains no product from a healthy control plant; lane 4 contains the T36 CP gene product amplified from a transformed sour orange plant carrying the T36 plant CP gene.

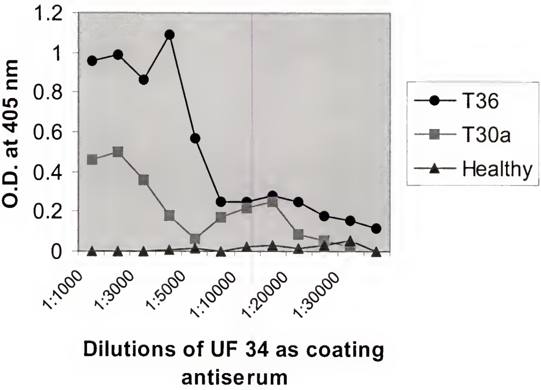
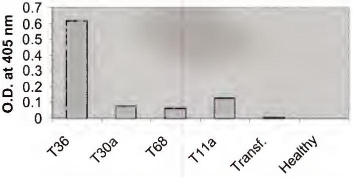
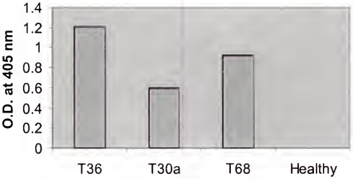


Fig. 4-2. Effect of the concentration of UF 34 specific for p27 capsid protein (CP) on detection of CTV isolates T36 and T30a. O.D. readings from DAS-indirect ELISA at 405 nm using extracts from plants infected with CTV isolates T36 and T30a, using UF 34 as the coating antisera at the dilutions indicated. G604 was used as the secondary antibody.



**Ability of UF34 to recognize the p27 CP of several CTV isolates**



**Ability of 1051 to recognize thel p25 CP of several isolates**

Fig. 4-3. Reactivity of UF 34 and 1051 for p27 and p25 coat protein (CP) respectively. Top) O.D.$_{405\ nm}$ readings from ELISA assays using UF34 at a dilution of 1:3000 for coating and extracts from plants infected with different CTV isolates as indicated, plus transformed plants expressing the T36 CP gene, and healthy plants as control. Bottom) O.D. readings from ELISA using extracts from plants infected with CTV isolates T36 and T30a using 1051 as coating antisera at the dilution of 1:1000. G604 was used as the secondary antiserum for all ELISA tests.

Table 4-1. Serological reactions of transformed plants and non-transformed control plants after inoculation with T36, T30a or T11a using UF 34 as coating antiserum in DAS-indirect ELISA. Top) ELISA reactivity using G604 as secondary antibody. Bottom) Specific detection by MCA 13 monoclonal of severe CP components.

| **UF 34/G604** | | **Transformed/sour** | | | **Sour/sour** | | |
|---|---|---|---|---|---|---|---|
| Controls | | T36 | T30a | T11a | T36 | T30a | T11a |
| T36 | **0.961** | **0.888** | **0.326** | **0.450** | **1.269** | **0.758** | **0.738** |
| T30a | **0.493** | **0.871** | N.d. | **0.207** | **1.059** | **0.625** | **0.787** |
| T11a | **0.377** | **0.614** | **0.367** | **0.285** | **0.638** | **0.727** | **0.550** |
| Transformed | 0.121 | **0.688** | **0.457** | **0.432** | **0.252** | **0.777** | **0.588** |
| Healthy | 0.084 | **0.827** | **0.302** | **0.358** | | | |
| Blank | 0.001 | **0.602** | **0.350** | **0.247** | | | |
| | | N.d. | **0.394** | **0.289** | | | |
| | | **0.623** | **0.268** | **0.265** | | | |
| | | **0.604** | **0.228** | **0.302** | | | |
| | | **0.589** | **0.287** | **0.259** | | | |
| | | **0.559** | **0.448** | **0.320** | | | |
| | | **1.072** | **0.271** | **0.317** | | | |
| | | **1.151** | **0.292** | **0.258** | | | |

| **UF 34/MCA13** | | **Transformed/sour** | | | **Sour/sour** | | |
|---|---|---|---|---|---|---|---|
| Controls | | T36 | T30a | T11a | T36 | T30a | T11a |
| T36 | **0.593** | **0.348** | 0.074 | **0.667** | **0.736** | 0.134 | 0.122 |
| T30a | 0.120 | **0.393** | N.d. | 0.158 | **0.293** | 0.106 | 0.089 |
| T11a | 0.059 | **0.253** | 0.172 | 0.113 | **0.336** | 0.144 | 0.088 |
| Transformed | 0.124 | **0.338** | 0.116 | **0.419** | **0.470** | 0.110 | 0.103 |
| Healthy | 0.125 | **0.545** | 0.061 | 0.004 | | | |
| Blank | 0.003 | **0.478** | 0.043 | 0.065 | | | |
| | | N.d. | 0.112 | 0.041 | | | |
| | | **0.525** | 0.025 | 0.104 | | | |
| | | **0.401** | 0.004 | 0.078 | | | |
| | | **0.397** | 0.048 | 0.033 | | | |
| | | **0.408** | 0.110 | 0.118 | | | |
| | | **0.530** | 0.035 | 0.112 | | | |
| | | **0.515** | 0.049 | 0.043 | | | |

N.d.= Not determined.

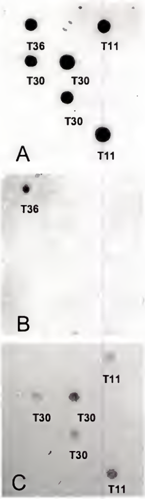


Fig. 4-4. Hybridization with strain specific probes of IC-RT-PCR products obtained from T11a samples positive to MCA 13 from transformed plants expressing p25 CP following inoculation with T11a. A) Probe 0 (universal). B) Probe I (quick decline). C) Probe VI (Florida mild).

0 (universal) and probe VI (Florida mild) but not with probe I (decline). This suggests that these two plants contained T11a genomes which had been heterologously encapsidated with the severe strain T36 CP being expressed by the transformed plant (Fig. 4-3).

## Discussion

The UF 34 antiserum which specifically immuno-captures the p27 minor CP was used for studying the heterologous encapsidation by the major p25 CP expressed in transformed citrus plants carrying the p25 CP gene of the T36 isolate and infected with virions of T30a , T11a, or T36 by graft inoculation. The antiserum UF34 shows a higher affinity for its homologous antigen, the T36 p27 CP, than for other CTV isolates, but it is still able to immuno-capture the p27 CPs from two other mild isolates, T11a and T30a, and the stem pitting isolate T68 (Table 4-1). Transformed plants expressing T36 major CP p25 were not serologically reactive with UF 34 since these plants do not express the minor p27 CP.

The CTV virions are encapsidated by two CP components, the p25 or CP, and the p27 or minor CP, arranged in a polar structure where by the p27 is located at one end and protects the 5'- terminus. The remainder of the genomic structure is encapsidated by the major CP p25 (chapter 3). Immuno-capturing CTV virions by the p27 CP allowed detection of particles of heterologously encapsidated p25 CP expressed by the transformed host. Furthermore, the specificity of the monoclonal antiserum MCA13 for T36 p25 CP and not for the p25 CP of T11a and T30a (Rocha-Peña *et al.* 1991; Permar *et al.* 1990) was exploited in this study. Two plants of the 13 plants tested which had been inoculated with T11a contained virions which reacted positively to MCA13. This suggests those virions were encapsidated with the CP expressed by the transgenic host. Additionally, it was demonstrated by the hybridization of the IC-RT-PCR products of the virions captured by the p27 with probes 0 and VI in the SSP analysis that they were not a product of a mixed infection with the severe isolate T36. This provides further evidence that the virions in the two T11a inoculated plants were heterologously encapsidated with p25 CP expressed by the transformed host. This phenomenon occurred at a low frequency considering that only two out of 13 plants, or two of 26 plants if considering the T30a inoculated plants, but a high frequency should be pondered if similar results extended to the whole citrus industry. Perhaps production of p27 antiserum specifically against T11a and T30a would enable a more precise study of the frequency of this event, because the UF34 antiserum used in this study appears to work best to detect T36 p27 (Table 4-1). It is not possible determine with the existing antisera whether the observed heterologous encapsidation was partial (phenotypic mixing) or complete (transcapsidation). The MCA13 did not work for coating in ELISA tests nor for serological specific electron microscope decoration using gold-conjugate antibodies.

Heterologous encapsidation, even at a low frequency, might favor a non-genetic variability of the CP elements. While the interactions of CTV and its vector are poorly understood at the molecular level, the consequences of heterologous encapsidation on virion transmission by vector is unpredictable. A more in-depth study of the impact is required. To prevent heterologous encapsidation as a possible enviromental risk, the use of partially deleted CP sequences should be considered for future transformation experiments.

# APPENDIX A IMMUNO-CAPTURE RT-PCR OF CTV FROM CITRUS

This protocol enables the detection of CTV genes and the serological quantitation of CTV coat protein by using both monoclonal and polyclonal antibodies. The use of MCA 13 for selective detection of severe strains of CTV or use of polyclonal antibodies for universal trapping of all CTV strains allows great versatility with this procedure in standard ELISA microtiter plates or directly in PCR microcentrifuge tubes. It is possible also to capture the virus directly and then proceed immediately to amplification of the gene without completing the ELISA reaction.

IMMUNO-CAPTURE (ELISA).

1. **Coating**

   Coat the plates or tubes with the primary antibody at the recommended concentration. Incubate 2-4 hours at 37 ºC or 4 ºC overnight.

   *We have been using CREC 31,CREC 35, 1051, 1052 and UF 34 antiserum successfully. Unfractionated antiserum may also be used.*

   Wash 3 times, 5 minutes each with PBS-0.05 % Tween. or PBS-Tr-Suc (PBS plus 0.2%Triton X-100 and 5% Sucrose.) for keeping the integrity of long flexuous particles.
2. **Sample preparation and CTV extraction**

   Grind 0.2-0.3 g of fresh bark in liquid nitrogen and add 1 ml of coating buffer or extraction buffer (PBS-0.05 % Tween- 2 % PVP [Polyvinylpyrrolidone]). Incubate 15-20 min. at room temperature.
3. **Trapping the virus**

   Pipette 100 µl of sample to each well or tube and incubate 2-4 h at 37 ºC or overnight at 4 ºC. Wash 3 times, 5 minutes each with PBS-0.05 % Tween. *Wash carefully to avoid cross contamination among wells.*

   If the serological quantitation by ELISA is not critical in your experiment, then go straight to step 8.
4. **Secondary Antibody**

   Dilute the selected monoclonal or polyclonal antiserum at the suggested concentration, MCA13, G604, 3DF1+ 3CA5 among others in conjugate buffer (PBS-0.05 % Tween ; 0.2 % bovine serum albumin [BSA]), load 100 µl to each well or tube, and incubate 2-4 h at 37 ºC or 4 ºC overnight. After incubation wash 3 times, 5 minutes each with PBS-0.05 % Tween.
5. **Conjugate reaction**

   Dilute the antigoat-, antimouse- or antirabbit-conjugate (according to antibody used in your experiment) in conjugate buffer (PBS plus 0.05% Tween; 2% PVP; 0.2 % BSA) using the recommended concentration (~1:30,000). Incubate 3-4 h at 37 ºC or overnight at 4 ºC. Then wash 3 times, 5 minutes each with PBS-0.05 % Tween.
6. **Substrate and reading**

   Develop the ELISA reaction as usual by pipetting 100 µl of substrate per well or tube. *We use coating buffer to dissolve 5-7.5 mg of PNP(4-Nitrophenyl phosphate); both 10 % diethanolamine and the coating buffer have the same pH.*

   Record the ELISA reaction reading, then wash 3 times, 5 minutes each with PBS-0.05 % Tween. Dry as usual. The dry plate or tube may now be stored at –20 ºC, but not longer than a week before the RT-PCR reaction takes place.

7. **RT-PCR**
   First remove the plate from of the freezer and let it warm to room temperature.
8. **Virus disruption buffer preparation.**
   The mixture may be prepared for 100, 50 or 25 µl to minimize operational costs.

**For 50 µl**

| | |
|---|---|
| 10X PCR buffer | 5 |
| 0.1 DTT | 5 |
| 25 mM MgCL2 | 5 |
| 10 mM dNTPs | 0.5 |
| Primer 1 | 1 |
| Primer 2 | 1 |
| Rnase free water. | 27.5 |
| Total | 45 µl |

Add the 45 µl of the virus disruption buffer to the well or tube containing trapped virus, and incubate at 70-75 °C for 5 minutes and let cool at room temperature. Then go to step 9 and add the prepared enzyme mixture.

9. **Enzyme mixture preparation**

| | |
|---|---|
| Rnasin (Rnase inhibitor) | 0.25 |
| Reverse transcriptase M-MLV[1] | 0.5 |
| Taq. DNA Polymerase[2] | 0.25 |
| Rnase free water. | 4 |
| Total | 5 µl |

[1] AMV (0.5 µl) or Superscript 0.25 µl may be used.

[2] Taq DNA pol. may be added after the RT reaction.

10. Add 4.75 µl or 5 µl of the enzyme mixture(depending on your choice at step 9) into each well or tube and incubate the 45 minutes at 42 °C. *Mineral oil may be added to minimize evaporation, keeping stable the molarity of the reaction.*
11. Add Taq DNA pol. if it wasn't added at step 9. Transfer the 50 reaction from the ELISA plate into clean PCR tubes if the immunocapture and RT were performed in ELISA microtiter plate.
12. Set up your PCR reaction and perform PCR as usual. We use the following settings for CTV-CP gene amplification. (Primers CN150 and CN 151).

| | | |
|---|---|---|
| Step 1 | 94 °C | 1 min. |
| Step 2 | 94 °C | 1 min. |
| Step 3 | 50 °C | 1 min. |
| Step 4 | 72 °C | 1 min. Back to step 2 x 39 cycles. |
| Step 5 | 72 °C | 10 min. |
| Step 6 | 4 °C | (Pause) |

13. Check for PCR products by running 10 µl of each reaction on a 0.8-1% agarose gel electrophoresis. If the expected product is present, the samples are then ready for further analysis or experimentation, such as SSCP analysis, cloning and sequencing.

## Buffers for ELISA

### 0.05 M Carbonate buffer, pH 9.6 (Coating buffer).

| | |
|---|---|
| $Na_2CO_3$ | 1.59 g |
| $NaHCO_3$ | 2.93 g |

Make up to 1 liter and store at 4 °C

### Phosphate buffered saline (PBS), pH 7.4.

| | |
|---|---|
| Na Cl | 40 g |
| $KH_2PO_4$ | 1 g |
| $Na_2HPO_4$ | 5.75 g |
| KCl | 1 g |

Make up to 1 liter, if ½ liter then 10X and store at 4 °C

### Washing buffer (1X PBS-T).

| | |
|---|---|
| 1X PBS | 1000 ml |
| Tween 20 | 500 µl |

### Enzyme conjugate buffer.

| | |
|---|---|
| 1X PBS-T | 1000 ml |
| Bovine Serum Albumin 0.2% | 0.2 g |

It is possible to use coating buffer as 'tissue extraction buffer' and ' substrate buffer' with good results.

References.

1. **Nolasco, G., V. de Blas Torres, and F. Ponz.** 1993. A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and sub-viral pathogens. J. Virol. Methods 45:201-218.

# APPENDIX B INFECTIOUS CTV PURIFICATION PROCEDURE

**Stock solutions:** (Store all at 4°C)

- 25% (w/v) sucrose in 0.05 M Tris, pH 8.0 (12.5 g / 50 ml)
- 40% (w/v) sucrose in 0.05 M Tris, pH 8.0 (20 g / 50 ml)
- 60% (w/v) sucrose in 0.05 M Tris, pH 8.0 (30 g / 50 ml)
- Stock solution of 20% (v/v) triton X-100 in water.
- 3.0 **molal** stock solution of $Cs_2SO_4$ in 0.05 M Tris buffer, pH 8.0.

  (27.15 g / 25 ml ; 54.3 g / 50 ml ; 108.6 g / 100 ml)
- Dilute the 3.0 **molal** stock with 0.05 M Tris buffer, pH 8.0 to make stock solutions of 1.5, 1.0 and 0.5 molal.
- Use autoclaved Tris 0.05 M and water if further RNA tests will be done after purification.
-

**1st day**

- Harvest tender bark tissue, it should be stripped, chopped into pieces of about 1 cm in length, and stored at –80°C until use.
- Autoclave all required glassware (syringes, grad. cylinders, mortars, beakers, JA-10 and JA-20 tubes, cheesecloth)

**2nd day**

- Pulverize the tissue using liquid nitrogen in a mortar and pestle. Optimum amount of tissue is about 40 grams tissue.
- Add powdered tissue to 160 ml of 0.05 M Tris buffer, pH 8.0, containing 0.1% 2-mercaptoethanol (160 μl).
- Filter the homogenate through 4 layers of cheesecloth, cfg. 8,000 rpm for 10 minutes at 4-10 °C (BECKMAN J2-21 / JA-10 rotor), collect the supernatant.
- Add 1.6 ml of the 20% Triton X-l00 stock solution to the supernatant, and stir gently with a glass rod.
- In SW28 tubes, add 21 ml of the virus extract,
- then insert funnel tubes, add 5 ml of the 25% sucrose stock solution,
- then add 5 ml of 60% sucrose stock solution. Balance with 0.05 M Tris buffer, pH 8.0.
- Cfg 15 hours at 12 °C at 19,000 rpm. Schedule to start centrifugation about 5-5:30 PM, so it will be finishing 8-8:30 AM on the following working day.

**3rd day**

Remove tubes from cfg. Using thin tubing on a glass barrel syringe, remove 1.5 ml aliquots from the bottom of each tube, remove **3** x 1.5 ml aliquots from each tube. Pool all bottom fractions and discard them. Keep the **2nd** and **3rd** fractions, pool all fraction number 2's and pool all fraction number 3's.

- Dialyse during the day against 0.05 M Tris, pH 8.0. Tie dialysis bags tight to prevent a large gain in volume.

- Remove from dialysis, and cfg 10 min at 4°C at 8000 rpm. Beckman J2-21 with JA-20 rotor.
- Bring the recovered volume to 28 ml using 0.05 M Tris buffer, pH 8.0.
- Mix (very well) with 12 ml of 3 molal $Cs_2SO_4$ in VTi5O tube (or type 60). Balance tubes well using 0.05 m Tris buffer, pH 8.0.
- Cfg. about 20 hr at 48,000 (reduce speed if using Type 60 rotor) at 4 °C. Beckman L7 ultracentrifuge and VTi50 rotor.

### 4th day

Collect bands (should be about 3/4 of the way up the tube) using syringe with thin tubing and bottom source of light. Collect top band (mostly fragmented CTV) first, then collect lower band (mostly intact CTV) using a different syringe. RT-PCR of top bands do not retrieve products)

### 5th day

- Dialyse against two changes of 0.05 M Tris buffer, pH 8.0 at 4 °C, over the next 12-24 hrs. Then select step A or B as follows

### 6th day

A. Preformed step gradients of $Cs_2SO_4$

Cfg 8,000 rpm for 10 minutes. The preparation can then be run on a second cycle of $Cs_2S0_4$ in preformed step gradients if greater purity is desired. Preformed step gradients: using the funnels in SW41 rotor tubes, add 6 ml sample prep, then 1.5 ml 0.5 **molal** $Cs_2SO_4$, then 1.5 ml 1.0 molal $Cs_2SO_4$, then 1 ml of 1.5 molal $Cs_2SO_4$. Cfg 36,000 rpm overnight at 4 °C, collect virus bands using a syringe with thin tubing.

B. Rate zonal centrifugation

Cfg. 38,000 rpm for 3 hours in Beckman L7 ultracentrifuge and SW41 rotor. Prepare 10 to 40 % gradients of sucrose (a day before) from 40% stock as follow further fractionation of intact and broken particles is wanted:

Set up six 13.5 ml SW-41 ultracentrifuge tubes (Beckman) with narrow glass funnels and add 1.5 ml of each 10 to 35 % sucrose. Pour only 1 ml for 40 % sucrose stock. Starting at 10%. Let the tubes set overnight at 4 °C to allow gradient to form.

Prepare 10 to 40 % sucrose stocks as follow:

For 40 % sucrose stock dissolve 20 g sucrose in 50 ml Tris 0.05 M pH 8.0.

10 % 3.0 ml 40% sucrose + 9.0 ml Tris 0.05 M pH 8
15 % 4.5 ml 40% sucrose + 7.5 ml Tris 0.05 M pH 8
20 % 6.0 ml 40% sucrose + 6.0 ml Tris 0.05 M pH 8
25 % 7.5 ml 40% sucrose + 4.5 ml Tris 0.05 M pH 8
30 % 9.0 ml 40% sucrose + 3.0 ml Tris 0.05 M pH 8
35 % 10.5 ml 40% sucrose + 1.5 ml Tris 0.05 M pH 8
40 % 1.0 ml 40% sucrose + 0.0 ml Tris 0.05 M pH 8

Fractionate the tubes by 0.4 ml fractions, and hold on ice. Fractions may be checked for CTV presence by ELISA, electron microscopy or by IC-RT-PCR.

*An ISCO Density Gradient Fractionator Model 640 was used, but gradients may be fractionated by piercing the bottom of the tube with a syringe needle and collecting droplets.*

References.

1. **Bar-Joseph, M., D. J. Gumpf, J. A. Dodds, A. Rosner and I. Ginzberg.** 1985. A simple purification method for citrus tristeza virus and estimation of its genome size. Phytopathology 75 :195-198.
2. **Lee, R. F., S. M. Garnsey, R. H. Brlansky, and A. C. Cohen**. 1987. A purification procedure for enhancement of citrus tristeza virus yields and its application to other phloem-limited viruses. Phytopathology 77:543-549.
3. **Garnsey, S.M., R. F. Lee and R. H. Brlansky.** 1981a. Rate zonal gradient centrifugation of citrus tristeza virus. Phytopathology 71:875
4. **Garnsey, S.M., R. F. Lee and R. H. Brlansky.** 1981b. Preparation and stability of infectious citrus tristeza virus (CTV). Phytopathology 71:218

## APPENDIX C CTV RNA FROM SUCROSE GRADIENT FRACTIONS

This protocol allows retrieving the total RNA from a virus-purified fraction collected from a 10-40 % sucrose gradient. We use a density gradient fractionator ISCO model 640, and collected 400 µl samples. It is not recommended to extract from lesser volumes, work in a cold room or keep reagents and samples on ice. We also fractionate the gradient at 4 °C.

1. Add 10 % SDS to the 400 µl gradient fraction to a final concentration of 0.5%
   (20 µl of 10 % SDS to 400 µl of purified-CTV sucrose fraction)
   Mix gently, no vortexing.
2. Heat for 10 min at 50 °C and centrifuge 1 min in a microcentrifuge.
3. Add phenol:chloroform (1:1.5 v/v). Mix, no vortexing.
   300 µl Phenol.
   300 µl Cloroform.
4. Centrifuge 5 min at 4 °C. Collect upper-RNA phase, and place it into a new sterile microcentrifuge tube.
5. RNA precipitation.
   Add to the recovered phase: 1 ml ethanol, and 50 µl sodium acetate 3M, pH 5.2.
6. Freeze samples at –80 °C for 1 hour or overnight *( best RNA yields overnight).*
7. Centrifuge 30 min at 4 °C, then pipette out alcohol carefully. *(Don't worry if you don't see any pellet).*
8. Let dry under vacuum at 4°C or ice for 10 min. It is convenient to wrap the tube-mouth with Parafilm® and perforate it with a clean needle to minimize contamination.
9. Resuspend in 10 to 20 µl of $H_20$ or DEPC water, and immediately aliquot according to your future experimentation usage to avoid unnecessary thawing that might reduce your RNA yield.
10. Store samples at –80 °C. Now, you are ready for cDNA, RT-PCR or Northern blotting procedures.

References.

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# APPENDIX D DESIGNING PRIMERS FOR PCR BASED EXPERIMENTS

This is a procedure intended to offer a software-internet-based strategy for primer design. The critical factors involved for a successful PCR are not discussed in this handout. For a better understanding of the basics of PCR primer design, it is recommended to read on the topic. Keep in mind the need to avoid unbalanced distribution of G/C and A/T rich domains and complementarily between 3' ends favoring the primer-dimer formation. Before starting, take a few minutes to download software, bookmark the suggested webpages and place the icons of 'Wordpad' and 'Rnadraw' on your desktop file. This will make it easier to paste and copy sequences between programs.

First Strategy:

**1. Get the sequence of interest from the gene bank.**

We use National Center of Biotechnology Information (NCBI) homepage.
http://www.ncbi.nlm.nih.gov/
Click on "Entrez", then upon "Nucleotide", type your keyword or the organism name at the prompt, and select the appropriate accession number of interest. Block and copy the sequence on which the primers will be designed.

**2. Go to the Baylor College of Medicine (BCM) Search Launcher page.**

The BCM Search Launcher is an integrated set of World-WideWeb (WWW) pages that organize molecular biology-related search and analysis services available on the WWW by function, and provide a single point-of-entry for related searches.
http://dot.imgen.bcm.tmc.edu:9331/seq-util/seq-util.html
Paste the formerly copied sequence into the working window (max. 7,000 bases). Convert in "fasta" format if necessary by clicking on "Read Seq" then "submit" on the same page. Click on "Primer selection" and submit. You will retrieve a reasonable list of forward and reverse primers that can be saved (on Wordpad) and printed for scratch-paper working or further analysis.

**3. Copy and paste your primers on "Wordpad".**

"Courier New" font, size 10, is recommended if complementary software like Genedoc, or ClustalX is going to be used for alignment analysis.

**4. Import your primer into "Rnadraw" (*recommended*) or go straight to step 5.**

Copy and paste from "Wordpad" to "Rnadraw", and calculate the secondary structure for each primer, one at time. "Rnadraw" software can be downloaded for free at
http://rnadraw.base8.se/ - wwwRnadrawStructureCalc/

Once in Rnadraw, go to "File" then to "New File". Write down the I.D. or name of your primer at the "File Title" window, and click on "Paste from Clipboard" and "OK".

Right click on "Sequence..." scroll down to "Calculate", click on "Structure", select the temperature (usually 50° C or above) then click on the "Calculate" box at the bottom leaf of the screen, and check your result. No secondary structure is observed at 0 Kcal. Right click again on "Secondary..." and scroll down to "2D radial drawing". Right click on the new "2D radial drawing" that shows up, and drag your cursor to "View".

At this point, clicking on the "Tile windows" icon over the upper toolbar allows a better view and management of the screen.

Moving the cursor inside the RNA structure, then left clicking on it increases the size of the structure. Right clicking decreases the size.

Moving the cursor to either side of the structure, and clicking on the left or right side allows for rotating the structure for a better positioning.

Moving the cursor to the bottom, the right click allows you to get the bases on the structure by checking the "Show Bases" box. Then click OK.

For printing, move the cursor to the top of the structure, and right click scrolling down to "Print".

It is possible that all retrieved primers (from step 2) contain internal hairpins. In that case, try skipping the primer sequences for a few bases or codons to avoid the complementarily region that induces the loop to form.

Now you have elements to get a better idea about the secondary structure of your primer at the selected temperature, and a final checking can be done.

5. **Go to Primers of the world homepage.**

http://www.williamstone.com/primers/index.html

In this website you can paste the sequence of your primer into the 'Oligo Calculator' to retrieve information on self-annealing scenarios, and to determine if annealing to other copies of themselves or reverse primers might occur.

Pasting the sequence of interest (from the genebank) into 'Primer Design' will generate an abundant list of forward and reverse primers. The primers can be analyzed as suggested in the previous 'Primer Design'" section.

Second Strategy:

A. **Get the sequence of interest from the gene bank** (same as step 1**).**

B. **Copy and paste your sequence of interest on Primer3 in fasta format** (you can convert in fasta as described in step 2**).**

http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

Identify your sequence at the "Sequence Id" box. Select your "targets" and "excluded regions". It is explained on the homepage how to do it. Fix some other general primer picking conditions on pages following the home page. Then click on "Pick primers" and wait for your primer output.

C. **Rules of the Thumb for primer design.**

- PCR primers are typically 18-25 nucleotides in length, with 40-60% GC content.
- In primer design, avoid complementary sequences of two or three bases at the 3' end of the primer pairs to prevent primer-dimer formation.
- Avoid internal complementary sequences to minimize internal hairpins.
- It is best not to have a 3'end T, or sequence runs of three or more Gs or Cs at that terminus.
- When introducing restriction sites or mutations into the amplification product, the bases that do not hybridize to the template may be added at the 5' end of the primer.
- Avoid mismatches between the 3' end of the primer and target-template sequence.
- For degenerate primers, it is preferable that the most unambiguous sequences be at the 3' end of the primer. The last three bases on the 3' end can be sufficient to initiate PCR.
- Annealing temperatures are usually set about 5 °C below the Tm of the primers. It is best if both primers have a similar Tm.

- Tm in the range of 55-70 °C gives the best results.
- Only use reaction tubes designed for PCR (thin wall). They must fit precisely into the block wells of your thermocycler.
- Check all the temperatures and times of each cycle step as well as the reactive and enzyme concentrations to be on the range expected for PCR amplification.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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